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THE EYES AND THE PHOTONEGATIVE BEHAVIOUR OF *NEPHTYS* (ANNELIDA, POLYCHAETA)

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I. INTRODUCTION

If *Nephtys* is dropped into a pool of water in the sand, it swims for a short time and then begins to burrow. The prostomium is thrust against the substratum and the undulatory waves passing along the body vibrate the prostomium in the sand and drive it forward. As soon as the anterior segments are buried, the locomotory activity changes to alternate contractions of the longitudinal and circular muscles and the worm burrows like an earthworm. Swimming is a necessary prelude to burrowing. Usually the worm buries itself at the first attempt, but if it does not do so, it remains inactive on the bottom of the pool for a time, perhaps a minute, and then swims and attempts to burrow again. The same pattern of behaviour is invariably followed: the worm swims at intervals and at the end of each swimming excursion attempts to burrow; the attempts are repeated until the worm does eventually bury itself. The experiments described below have been designed to provide at least a partial analysis of this behaviour on the supposition that one of the factors controlling it is the illumination of the worm.

Two reactions to illumination are found in the polychaetes; (*a*) a synergic contraction of the longitudinal muscles mediated by giant axons, or (*b*) locomotion. The former is characteristic of the tubicolous, the latter of the errant worms. These responses are not limited to those families of polychaetes conventionally regarded as 'sedentary' and 'errant' respectively, but the sudden contraction response is more pronounced in worms living in permanent or semi-permanent burrows and tubes, and the locomotory response is more conspicuous in worms which do not inhabit burrows. The phenomenon of contraction is the more spectacular and is more amenable to experimentation, and it has accordingly attracted more attention than the locomotory response. The literature on the responses of tubicolous polychaetes to stimulation by light has been discussed and reviewed by Nicol (1950). Among the polychaete families usually regarded as 'errant', the Nereidae is the only one in which the response to illumination has been studied in detail. Herter (1926) found that *Nereis diversicolor* is negatively phototactic. However, even the nereids usually live in consolidated burrows or secreted tubes of a more or less permanent character and they contract on a sudden change of light intensity, whether it be an increase or a decrease. Several polychaete families include species which are pelagic during the breeding season, and numerous observations scattered in the literature indicate that these worms are photopositive at this time at least, in

so far as they are attracted to lights in the water. This represents a reversal of the usual photic response, but there appears to have been no systematic investigation into the precise mechanism of this behaviour.

Nephtys differs from those worms which have been studied in detail in this context, in that although it burrows in the sand, it does not form a permanent, consolidated burrow, and the sudden withdrawal on stimulation which is so well marked in tubicolous worms is not found to any marked degree in the Nephtyidae. Both its habits under natural conditions and the observations of its behaviour in the laboratory suggest that it is much closer to the generalized 'errant' polychaete than any that has been studied before.

II. THE STRUCTURE AND DISPOSITION OF THE PHOTORECEPTORS

Most taxonomic and morphological accounts of the Nephtyidae, e.g. those by McIntosh (1908) and Fauvel (1923), mention the presence of a pair of eyes in *Nephtys*, but fail to describe them. There are numerous references in the literature to pigment spots and ocelli on the prostomium, presumably intended to suggest the presence of epidermal photoreceptors, and these are also without descriptions. What few descriptions there are do not inspire confidence. Ehlers (1864–8) refers to a pair of refractile, lens-like structures in the posterior part of the prostomium and Schack (1886) to a pair of round eyes on protuberances at the posterior margins of the prostomium. Neither of these observations is correct. Schack, and possibly also Ehlers, mistook the nuchal organ for eyes and, of the nineteenth-century anatomists, only Claparède (1868) certainly saw them. Hanström (1928) dismisses them with the remark that when eyes are present they are embedded in the brain and are simple pigmented ocelli. The eyes of *Nephtys* have thus never been the subject of any but the most cursory examination.

Three types of photoreceptor are found in members of this family, though not all species possess all three types. They are: (*a*) single-celled receptors lying in pigment cups and embedded in the supra-oesophageal ganglion, a pair of unit receptors on either side (these are the ocelli to which Hanström made reference); (*b*) single-celled receptors lacking pigment cups, situated in the prostomium at the extreme anterior edge of the supra-oesophageal ganglion; (*c*) epidermal receptors in the body wall, probably in the pygidium of two small species and possibly in the prostomium of several species.

Techniques

Specimens of *Nephtys* have been fixed in Bouin's fluid, picroformol, Heidenhain's 'Susa', Zenker-formol and Zenker-acetic, all made up in sea water. Small specimens have been fixed whole, but in larger specimens the anterior centimetre was cut off and fixed for 15–30 min., then partly dissected and returned to the fixative for a further period to improve penetration. Paraffin sections have been cut in the transverse and frontal planes at a thickness of 7 μ. The following staining techniques have been employed: Mallory triple stain, Heidenhain's iron haematoxylin, and the paraldehyde-fuchsin with trichrome counterstain technique (Clark, 1955).

Photoreceptors of the supra-oesophageal ganglion

Bilaterally symmetrical photoreceptors embedded in the posterior part of the supra-oesophageal ganglion are present in nearly all species of *Nephtys*. Only *N. incisa* among the species I have examined lacks them (see Table 1). The eyes are not generally visible from the exterior, nor even in dissection unless the ganglion happens to be cut at the right level. However, in two small species, *N. cornuta franciscana* and *N. parva*, they are visible from the exterior by transparency (Clark & Jones, 1955, figs. 1, 2). The eyes of these species are about half the size of those of a large species, such as *N. californiensis* or *N. caeca*, but the linear dimensions of the worms themselves are reduced by a factor of 20–25, and the amount of tissue

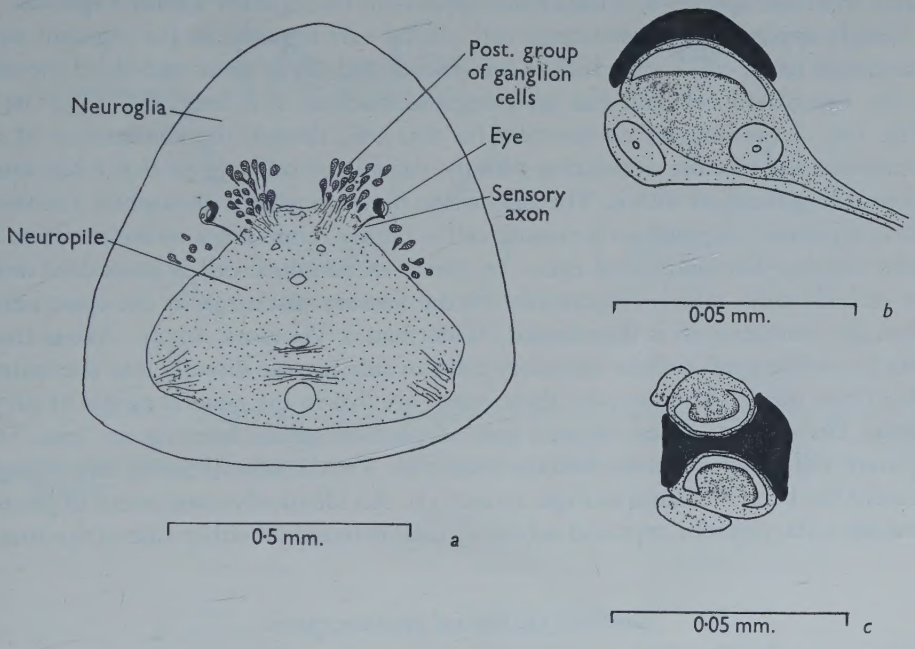

Fig. 1. *a.* Frontal section through the supra-oesophageal ganglion of *N. californiensis* showing position of the posterior eyes. *b.* Sagittal section through a single element of the photoreceptors of *N. californiensis*. *c.* Transverse section through the photoreceptors of *N. ferruginea*.

overlying the eyes is both relatively and absolutely very much less. In these two small species the eyes can be seen as two black spots through the dorsal side of the third segment, the supra-oesophageal ganglion extending from the prostomium to that segment. In all species of *Nephtys* which possess eyes, these lie in the posterior part of the brain, a little anterior to two paired groups of ganglion cells at the postero-lateral corners of the neuropile (Fig. 1*a*).

Each photoreceptor consists of a single vacuolated cell lying within a pigment cup which is composed of dark brown (melanin?) extracellular granules. Since there are no other cells in the immediate neighbourhood, apart from dense, coarse neuroglial fibres, it seems likely that the pigment granules are secreted by the

sensory cell itself. The dimensions of each photoreceptor cell are about 40 × 20 μ in the largest worms, such as *N. californiensis* and *N. caeca*, and about half that size in the smallest species. In other species they are intermediate in size. Projecting into the vacuole of the cell is a ridge which is mushroom-shaped in cross-section (Figs. 1*b*, *c*). Photosensitive material is probably concentrated in this ridge, particularly at its surface, since it possesses somewhat different staining properties from the rest of the cell. The almost spherical nucleus is 8 or 9 μ in diameter and lies near the origin of the axon. Two such vacuolated cells lie close to each other and their pigment cups are contiguous, resulting in an H- or Y-shaped mass of pigment with the receptor cells directed either dorsally and ventrally or else dorso-laterally and ventro-laterally. The particular form of the composite pigment cup varies from one species to another but appears to be constant within a species.

Closely applied to the vacuolated cell, on the side opposite to the pigment cup, is a second nerve cell body which is non-vacuolated. It is about one-third the size of the vacuolated cell and has an elongated nucleus 15 μ long and 5–7 μ wide (Fig. 1*b*). I can suggest no function for this cell, though the consistency of its occurrence and its close association with the vacuolated cell suggest that it has some functional association with it. The association between what is apparently a sensory cell in a pigment cup and an accessory cell is faintly reminiscent of the situation in some arthropodan compound eyes. In these, an eccentric cell is associated with the retinula cells, and is responsible for the sensory discharge in the optic nerve when the ommatidium is illuminated (Waterman & Wiersma, 1954). Axons from both the sensory cell and the accessory cell run side by side directly into the neuropile. Once inside the neuropile, these axons are lost in the general tangle of nerve fibres. The sensory axons of each pair of photoreceptors forming an 'eye' run separate, but parallel, courses into the neuropile. For the sake of clarity only a single element has been indicated in Figs. 1*a* and 1*b*. An identical arrangement of photoreceptor cells, pigment cups and accessory cells is found on either side of the brain.

Anterior prostomial photoreceptors

Two pairs of cells, which are probably photoreceptors, lie in the anterior part of the prostomium outside the supra-oesophageal ganglion. They are not universally present, nor do they always have the same appearance. *N. incisa*, *N. punctata* and *N. rickettsi* appear to lack them, while in the remaining species they may be vacuolated and resemble the eyes in the supra-oesophageal ganglion closely, or they may be non-vacuolated (see Table 1). There is little to show that the non-vacuolated cells are photoreceptors, but whether functional or not they are assumed to be homologous with the vacuolated anterior receptors of other species because they are the same size and shape, are in the same position and bear the same relation to the brain.

The circum-oesophageal connectives leave the supra-oesophageal ganglion at its antero-lateral corners. Immediately dorsal to the connectives there is a group of small ganglion cells extending from one side of the ganglion to the other (Fig. 2*a*).

The prostomial photoreceptors lie immediately anterior to these cells, in contact with, but internal to the epidermis of the lateral walls of the prostomium. They are about $40 \times 20\,\mu$ in size and have an oval rather than a spherical nucleus. In those species in which they are vacuolated a ridge projects into the vacuole of the cell, and there is some indication that the photosensitive material is concentrated in the surface of the ridge (Figs. 2*b*, *c*). These cells invariably lack pigment cups, but

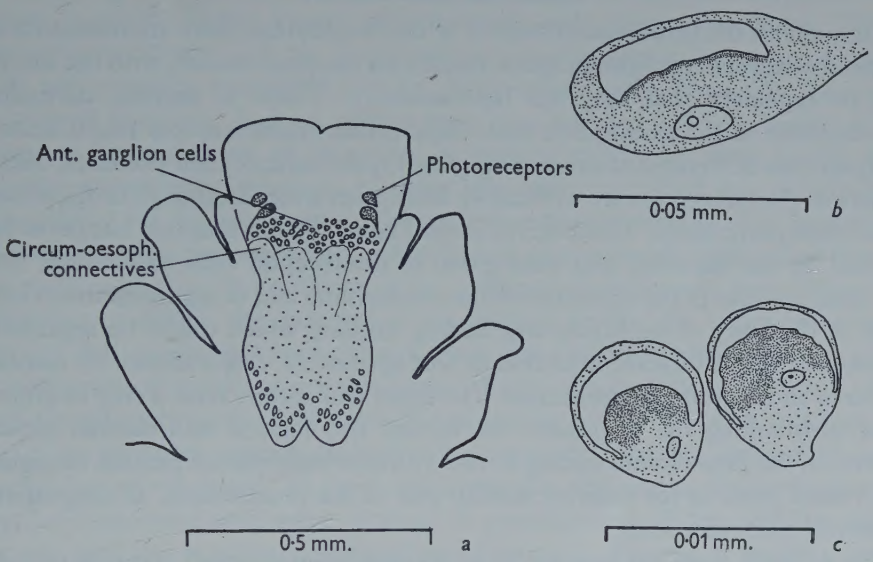

Fig. 2. *a*. Frontal section through the prostomium and anterior segments of *N. cirrosa* showing the position of the anterior eyes. *b*. Sagittal section through a single element of the anterior photoreceptors of *N. californiensis*. *c*. Transverse section through the anterior eyes of *N. ferruginea*.

Table 1

Species	Anterior eyes	Posterior eyes
N. caeca	non-vacuolated	present
N. caecoides	vacuolated	present
N. californiensis	vacuolated	present
N. cirrosa	non-vacuolated	present
N. cornuta	non-vacuolated	present
N. cornuta franciscana	non-vacuolated	present
N. ferruginea	vacuolated	present
N. hombergi	?	present
N. incisa	?	?
N. longosetosa	vacuolated	present
N. magellanica	vacuolated	present
N. parva	vacuolated	present
N. picta	vacuolated	present
N. punctata	none	present
N. rickettsi	none	present
N. squamosa	non-vacuolated	?

otherwise the vacuolated variety is almost identical with the photoreceptors of the supra-oesophageal ganglion; indeed, it is chiefly for this reason that they are presumed to be photoreceptors. The non-vacuolated cells appear to be homologous

with the vacuolated ones. There is no suggestion of an accessory cell in contact with the photoreceptor, but usually two identical photoreceptor cells lie in contact with each other. The axons from the photoreceptor cells on either side of the prostomium run laterally and caudally and enter the supra-oesophageal ganglion at its anterior margin at about the point of origin of the circum-oesophageal connectives.

Epidermal photoreceptors

Throughout the taxonomic literature of the Nephtyidae there are numerous and repeated references to pigment spots, usually on the prostomium, with the implication or statement that they are light-sensitive. There is nothing particularly reprehensible in the assumption that single-celled photoreceptors might occur in the epidermis of *Nephtys* and that the pigment spots indicate their presence, because epidermal photoreceptors are well known from other annelids, though in oligochaetes rather than polychaetes. However, as far as I know, this assumption has never been justified by the discovery and description of the receptor cells themselves. After searching sections of the epidermis of the prostomium and of segments from various parts of the body of nephtyids and finding nothing which might be regarded as a photoreceptor, I directed attention to four species, *N. californiensis*, *N. caecoides*, *N. parva* and *N. cornuta franciscana*. The latter two species have a ring of pigment spots surrounding the pygidium, the former two have a well-marked pigment pattern on the prostomium ending in two symmetrically placed patches of pigment and a third patch in the anterior median part of the prostomium, all suggestive of epidermal photoreceptors.

The pigment spots are formed by an accumulation of small granules of a dark brown substance which are extracellular and which surround certain epidermal cells. Apart from the fact that they are surrounded by pigment granules, these cells differ in no way from other epidermal cells in the neighbourhood. They are roughly conical in shape, with the base of the cone distal and the apex in contact with the basement membrane of the epidermis. Epidermal photoreceptors of other annelids are usually differentiated from structural epidermal cells by the presence of vacuoles or refractile bodies, and the fact that some epidermal cells in *Nephtys* are surrounded by pigment seems an insufficient reason for regarding them as photoreceptors. Particularly in the two small worms, *N. cornuta* and *N. parva*, pigment granules are laid down along practically all the connective tissue of the body and the epidermal cells of the pygidial ring differ from the others only in that the pigment granules surrounding them are more numerous and more concentrated. On the other hand, all four species react by swimming or by a slight contraction of the longitudinal musculature if light is shone on the posterior segments; the middle of the worm appears to be insensitive to light. The same is true of *N. caeca*, *N. cirrosa*, *N. hombergi* and *N. punctata*. If there are any photoreceptors in the epidermis, they are cells of this type and are not specialized morphologically. It is possible, though I have not been able to observe it, that fibres from these cells run through the basement membrane of the epidermis to connect with a sub-epidermal nerve plexus.

III. EXPERIMENTAL STUDIES

The experiments fall into two groups: first, a series of investigations into the nature of the photonegative reactions of a single species, *N. cirrosa* Ehlers, and secondly, an investigation of the function of the anterior prostomial and the ganglionic photoreceptors. Ideally the latter investigation should be carried out by removing one or other pair of eyes and observing the resulting changes of behaviour in the manner in which Herter (1926) experimented with *Nereis diversicolor*. This is impossible in *Nephtys*, for the single-celled receptors cannot be removed individually and could be extirpated only by doing considerable damage to the supra-oesophageal ganglion. Instead, species have been selected for these studies which possess one or other set of receptors, or both of them. In other words, by a fortunate interspecific variation in these eyes, the extirpation has already been accomplished for the investigator. For such experiments to be valid, it is necessary to assume that the brain structure is identical in all species of *Nephtys* and that the only variable in these experiments is the presence or absence of eyes. Since it is impossible to follow the interneural connexions within the supra-oesophageal ganglion, this assumption is gratuitous, even if at first sight reasonable in a group of closely related species of a single genus. Even on a cursory examination of the supra-oesophageal ganglion of a number of species of *Nephtys*, it is apparent that the minute structure varies considerably from species to species, particularly in the anterior part of the brain. In these circumstances, the results of the experiments must be interpreted with great caution. A consideration of their validity will be deferred until the results are analysed and discussed. Because at sexual maturity there may well be changes in the behaviour of the worms when they are illuminated, only immature specimens of *Nephtys* have been used in this study.

Experiments with Nephtys cirrosa

(*a*) *The light-dark choice experiment.* When several worms are placed in a rectangular dish of water, one half of which is illuminated and the other half covered with a black screen, they eventually move into the darker half. This is a surprisingly lengthy process for so active an animal as *Nephtys*, and it is evident that the number of worms in each half of the dish fluctuates because worms re-enter the lighted half from the dark. If each worm is placed in a separate dish, but otherwise under the same experimental conditions as before, the number returning into the light half is reduced because in this case quiescent worms in the dark half are not disturbed by active worms as they accumulate there. Even so, although worms spend more time in the dark compartment than before, they still reappear in the light; it is only exceptionally that they are all in the dark compartment at the same time, and in no case do they remain permanently in the dark. Fig. 3 shows the results of two experiments. The lower line indicates the movements of thirty-eight worms distributed between three dishes and the upper one of seventeen worms, each in its own dish. The results show that *Nephtys* is photonegative, though they provide no information about the mechanism of the process. Further, they show that under these

experimental conditions, the orientation movement is imperfect because the worms do not stay in the dark when they reach it.

(*b*) *The effect of illumination on the activity of* Nephtys. If *Nephtys* is placed in a jar of sea water and is illuminated it swims around the vessel for a short time at fairly regular intervals. Other forms of activity in these circumstances have been observed but rarely, and it is concluded that normally the worms will exhibit alternating periods of rest and activity. This behaviour has been used to study the effect of illumination on the worms.

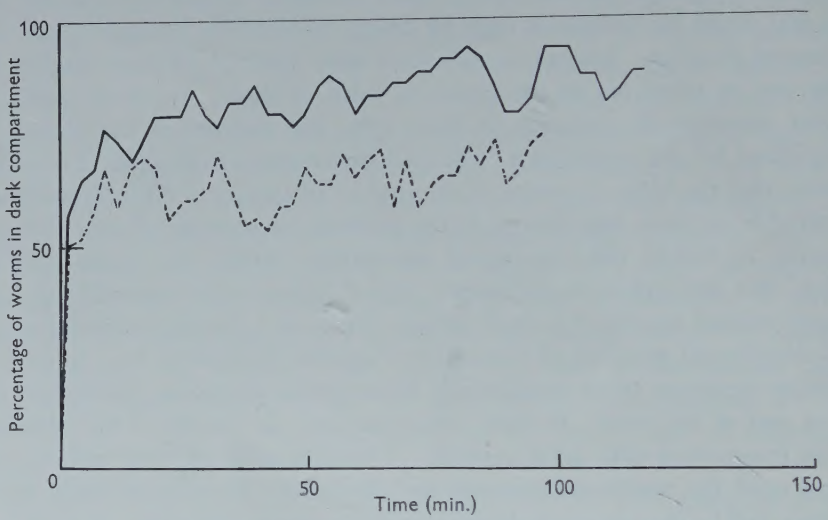

Fig. 3. Rate of movement of *N. cirrosa* from the light compartment to the dark in the light-dark choice experiment. Upper line, single worms; lower line, groups of worms in each experiment.

Illumination was provided by eight 60 W. 'Metrovik' electric light bulbs, rated at 250 V. and run at 245 V. from the public mains supply. The intensity was adjusted by varying the number of bulbs and the distance between them and the jars containing the worms. In this way the intensity could be varied without affecting the spectral quality of the light. During the period of the experiments the voltage of the mains supply varied by less than 3%, which is insufficient to affect either intensity or spectral quality appreciably. According to information supplied by the manufacturers, the spectral output of the bulbs is as shown in Table 2. A diffusing screen of a single sheet of tracing paper was interposed between the light and the worms. This affects slightly the spectral quality of the light falling on them, but this has not been measured. The intensity of the light was measured with a Weston photocell and meter and was adjusted in equal logarithmic steps between 0·45 and 14·4 candles per sq. ft. The worms, *N. cirrosa* Ehlers, were between 70 and 85 mm. long and were collected at low tide. Each was immediately placed in a glass jar containing about 750 cc. of fresh aerated sea water. The jars with the worms were kept in complete darkness for $2\frac{1}{2}$ hr. until the experiment was started. After this period of dark-adaptation the light was switched on, and for the

Table 2

Wavelength (Å)	Incandescent 60 W. Tungsten filament lamp, 2848° K.	Natural daylight, 6500° K.
3800–4200	0·0054	0·032
4200–4400	0·0585	0·26
4400–4600	0·249	0·83
4600–5100	5·39	10·65
5100–5600	33·52	41·8
5600–6100	42·68	35·8
6100–6600	16·5	9·9
6600–7600	1·52	0·68

following 2 hr. the activity of each worm was measured by counting the number of times it swam during that period. At the end of the experiment the water was changed in preparation for the next period of dark-adaptation and observation. All the experiments were carried out on the same individuals and were completed within 3 days of collecting the worms. The experiments were conducted in a concrete-floored room, free from vibration and sudden noise, with the water temperature constant at 14° C. The oxygen concentrations in the water, measured by the Winckler technique, did not change appreciably during the course of an experiment.

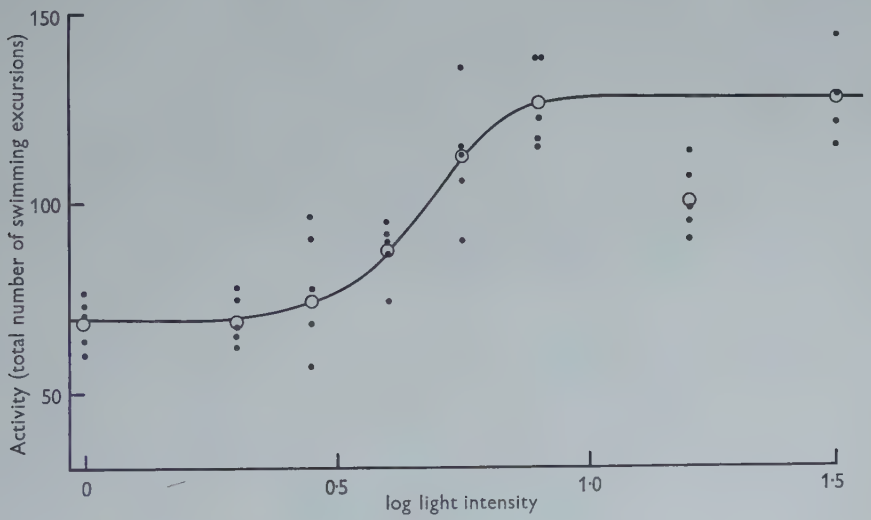

Fig. 4. Number of swimming excursions of *N. cirrosa* in two hours at various light intensities, following 2½ hr. dark adaptations.

Observations were made on five worms simultaneously and the results are shown in Fig. 4. It will be seen that there is a sigmoid relationship between the swimming activity of the worms and the logarithm of the light intensity, so that, within limits, the activity is approximately a linear function of the logarithm of the intensity, and outside these limits there is no change in activity with a change in light intensity.

No lower limit of light intensity below which the worms are inactive has been found. Even when illuminated with dim red light they are still active.

(*c*) *Orientation in a light beam.* A single worm was placed in a large glass tank of water lined on three sides and on the bottom with black matt paper to reduce light reflexion to a minimum. The worm was dark-adapted for half an hour and then illuminated from one end of the tank by a beam of parallel light from a 'Pointolite' microscope lamp and the behaviour of the worm was recorded. As soon as the worm reacted the light was extinguished and the worm left in darkness for a further period of half an hour. After eight or nine such tests the worm was discarded and a fresh one used. The light was always at the same end of the tank and always projected along the bottom of the tank. The direction at which the light beam impinged on the worm depended entirely upon the position taken up by the latter during the period of darkness. The conditions of the experiments are summarized in Table 3. With one exception, the reaction of the worm was always

Table 3

Direction of incident beam	Worm						Total exposures
	A	B	C	D	E	F	
Anterior	8	2	—	1	1	1	13
Posterior	2	1	1	2	4	2	12
Lateral	—	5	1	2	5	1	14
Post. diag.	—	1	1	1	—	—	3
Ant. diag.	—	—	—	—	—	—	0
Total exposures	10	9	3	6	10	4	42

the same; it swam forward even on the occasions when this involved swimming directly up the light beam towards the light source. In the single exceptional case the worm failed to react at all. The results of these experiments clearly indicate that whatever the direction of the incident light beam *N. cirrosa* does not orient itself in it.

(*d*) *Inhibition of the photonegative reaction.* Five worms were placed in jars of sea water containing enough sand to cover the bottom to a depth of 2–3 mm.; the sand was not uniformly distributed, but was heaped in some places to a depth of 5 mm. The worms were then exposed to light. Four of the worms, when they had burrowed as far as possible into the sand, showed no further activity, although most of the body was still exposed to light. In one case, where the sand was not heaped up and therefore nowhere deep enough for effective burrowing, the worm was more active than the others. The same worms, under the same conditions in jars without sand, were active during the whole period of 2 hr. during which they were under observation. The results are summarized in Table 4.

Four experiments were then carried out to test whether this inhibition of the reaction was brought about by the contact of the worms with the sand or by the obscuring of the light by it.

(1) Four worms were placed in glass tubes 20 cm. long and 4 mm. internal diameter in a tank of water and then illuminated.

(2) Six worms were provided with short lengths of glass tubing (15 mm. long), placed in water and llluminated.

Table 4. *Number of swimming excursions during 2 hr. exposure to light following 2 hr. of dark-adaptation*

Worm	With sand	Without sand
A	1	29
B	19*	37
C	1	47
D	1	65
E	3	29
Average	1·5	43

* Worm B was in a jar with insufficient sand for effective burrowing. The number of its swimming excursions has been omitted from the average figure.

(3) Six worms were placed between glass plates in a tank, so that the dorsal and ventral surfaces of the worm were in contact with the plates, and were then illuminated.

(4) Six worms were provided with dorsal contact over a short length of the body by placing them under microscope slides raised on supports.

In the first three experiments all activity was inhibited while the worms were illuminated for a period of 2 hr. following 2 hr. dark-adaptation. The fourth experiment was inconclusive because the worms did not stay in position under the microscope slides for any length of time. It follows that provided part, at least, of the dorsal and ventral surface of the body are in contact with some solid object the reaction to illumination is inhibited.

In the light-dark choice experiment, it was observed that three worms were inactive and remained in the light so long as they were upside down, i.e. ventral side uppermost. When they were righted they swam into the dark compartment within a short time. In another experiment of this kind, the worms lying upside down were left undisturbed, and the time they took to right themselves and swim into the dark compartment was observed and compared with the time taken by worms lying ventral side downwards (Table 5). In both these experiments the

Table 5. *Time taken by worms to reach the dark compartment in the light-dark choice experiment*

Lying ventral side down (min.)	Lying ventral side up (min.)
0·5	95
0·5	117·5
2·0	225
	11·5
	15
Average 1·0	92·8

worms were in separate dishes, so that disturbance by active worms swimming into quiet ones did not affect the results. Although the inhibition of the photonegative behaviour is only partial, and in any case *Nephtys* does not normally lie upside down unless it is moribund, there is a suggestion in these results that dorsal contact alone is sufficient to inhibit the activity of the worms in light and that ventral contact with the substratum, while usual, is superfluous. But the possibility remains that since the illumination was from above, the photoreceptors, which are nearer the

Table 6. *Time, in seconds, taken for worms to reach the dark compartment when suddenly illuminated from below*

Worm	Test no.					Average
	1	2	3	4	5	
A	14	17	12	23	23	18
B	9	20	16	—	—	15
C	173*	17	295*	22	—	127*
D	12	—	—	—	—	12
E	17	12	15	5	9	12
F	5	6	13	10	—	9
G	10	9	—	—	—	10

* Two swimming excursions before entering dark compartment.

dorsal than the ventral surface, were not adequately stimulated when the worm was upside down and the light struck the ventral surface. A series of experiments was therefore carried out in which the worms lay on the ventral surface, but were illuminated from below. If it is true that the opacity of the body is sufficient to prevent adequate stimulation of the photoreceptors, the worms should remain inactive although the dorsal surface of the worm is not in contact with any solid object.

Single worms were placed in a glass tank of sea water, of which half the bottom, the sides and most of the top were covered with black matt paper to reduce reflexion and to provide light and dark compartments when the tank was illuminated from below. The worms were left in darkness for half an hour, and then the light below the tank was switched on and the time taken for the worms to reach the dark compartment was recorded. All the worms were ventral side downwards during the experiment. The worms reacted to illumination by swimming and in all but two cases reached the dark compartment in a single swimming excursion. The two exceptional cases represent occasions when two swimming excursions were necessary. Thus the direction of the incident light is found to be irrelevant and the worms react as readily when they are illuminated from below as from above. This finding conforms with the possibility that dorsal contact alone is sufficient to inhibit the reaction to illumination. In addition, single-celled receptors, with a fine sensory hair projecting through the cuticle, have been detected on the dorsal surface of the worm. There are others on the ventral surface, but those on the dorsum are more numerous. These are presumably contact receptors, and thus the worm has the sensory equipment to detect contact between its dorsal surface and the sand.

Experiments with Nephtys cornuta

N. cornuta franciscana Clark & Jones has the same complement of photoreceptors as *N. cirrosa*, that is, there are paired receptors in pigment cups embedded in the supra-oesophageal ganglion and a pair of non-vacuolated cells without pigment cups in the anterior part of the prostomium. However, it is a very small worm and the eyes lie very close to the surface of the dorsum. In *N. cirrosa* the eyes are so deeply embedded in the ganglion that it is difficult to imagine that any but very diffuse light reaches them at all. This being so, it is not surprising that although *N. cirrosa* has bilaterally symmetrical photoreceptors, which by virtue of the pigment cups are directional, it should not be able to orient itself in a light beam. In *N. cornuta*, on the other hand, the eyes are so close to the surface that there is a likelihood that they are more perfectly functional. The following experiments were designed to test whether this is so or not.

Single worms were placed in a glass tank of sea water and illuminated from one end of the tank by a parallel beam of light from a microscope lamp. The worms were dark-adapted for half an hour before each test and some seventy tests were carried out on twelve specimens. As before, the direction of the incident beam depended on the position taken up by the worm between tests, but sufficient observations were made to cover all possible directions of incident light relative to the worm. Two types of reaction were observed. The worms sometimes swam forwards, gradually turning into the beam until they were orientated longitudinally with respect to it, and then swam down-beam. This was most often observed when the incident light was lateral or posterior. Usually when the worms were facing the light when it was switched on, they swam once or twice in a tight circle in the beam and then swam down it until they reached the end of the tank furthest from the lamp. On eighteen occasions the worms failed to react at all, on eight the worms swam out of the beam and did not appear to orient themselves, while on two occasions the worms circled and then swam into the beam towards the light. If worms were dropped from a pipette into the light beam, they invariably oriented themselves in it and swam down-beam. All this is clear evidence that *N. cornuta* is able to orient itself in a light beam and suggests that *N. cirrosa* does not do so because the eyes are too deeply embedded in the ganglion to be completely functional.

Experiments with Nephtys californiensis *and* Nephtys punctata

N. californiensis Hartman and *N. punctata* Hartman both differ from the two foregoing species in the structure of the eyes. *N. californiensis* has both anterior prostomial and ganglionic eyes, but the former are vacuolated. *N. punctata* has ganglionic, but no anterior prostomial eyes. Both species when placed in a tank with light and dark compartments find their way into the dark compartment, but reappear periodically in the light, as *N. cirrosa* does under the same circumstances. Tests were made on six specimens of *N. punctata* and eight of *N. californiensis* to find if either worm was able to orient itself in a light beam. The experimental technique was the same as that described previously. In ten of the forty-five tests

made on *N. punctata*, the worms failed to react when illuminated; on all the other occasions they swam forwards regardless of the direction of the incident light beam. The worms showed no sign whatever of orientation in the beam. The same results were obtained in thirty-six tests on *N. californiensis*, on three occasions the worms failed to react, on the rest they swam forward and did not orient themselves. Thus, although exhaustive tests have not been made, it is safe to assume that the morphological difference between the eyes of *N. cirrosa* and *N. californiensis* do not affect their function and that even when the anterior eyes are missing, as they are in *N. punctata*, the behaviour is not altered.

Conclusions

From the foregoing experiments, the following preliminary conclusions can be drawn about the functions of the eyes.

(1) No differences in behaviour can be detected between species with vacuolated and non-vacuolated prostomial photoreceptors (cf. *N. cirrosa* with *N. californiensis*), nor between species with anterior receptors of either type and those lacking them altogether (cf. *N. californiensis* and *N. cirrosa* with *N. punctata*).

(2) The photoreceptors of the supra-oesophageal ganglion, although theoretically adequate to permit the animal to orient itself in a light beam, are apparently too deeply embedded in the brain for this in most species. When the ganglionic photoreceptors are close to the surface, however, the worm can orient itself (cf. *N. cornuta* with *N. californiensis*, *N. cirrosa* and *N. punctata*).

(3) Even when deeply embedded in the supra-oesophageal ganglion, the posterior eyes apparently receive sufficient stimulation by diffuse light for the animal to perform kinetic orientation movements (cf. *N. punctata* with *N. californiensis* and *N. cirrosa*).

(4) No experiments have been carried out on a species of *Nephtys* which possesses anterior eyes, but no ganglionic eyes, nor is it known if any such species exists. Thus it is not clearly established whether the anterior prostomial receptors are functional or not. There are two possibilities. Either the anterior receptors are completely non-functional and the stimulation of the ganglionic receptors by diffuse light evokes kinetic orientation movements in the worm, or else they are functional and are involved in the kinetic movements, but they can be functionally augmented or even replaced by the posterior eyes.

IV. DISCUSSION

The photoreceptors of the brain and prostomium of *Nephtys* resemble those found in other annelids. The photoreceptors in the epidermis of *Lumbricus* consist of single, vacuolated sensory cells without pigment cups (Hess, 1925), while in *Stylaria* there are several such cells in a group on either side of the prostomium, each partly invested by small pigment cells (Hesse, 1902). The unit photoreceptor of leeches also consists of cells of this type, though usually several are grouped within the same pigment cup (Scriban & Autrum, 1932–4). Polychaete eyes are frequently

more complicated and may include more than one type of cell. However, the lateral ocelli of *Polyophthalmus* (Opheliidae) take the form of single, vacuolated cells of the type found in *Nephtys*, but with digital processes projecting into the vacuole (Hesse, 1899).

Judged by their disposition and their relationship with groups of ganglion cells in the brain, the eyes of *Nephtys* are homologous with those of *Nereis*. The sensory axons of the posterior receptors of *Nephtys* enter the neuropile immediately anterior to the postero-lateral ganglion cells (Fig. 1*a*), exactly as the posterior optic nerves do in *Nereis* (Scharrer, 1936, and personal observation). These ganglion cells are homologous in the two worms and both are important neurosecretory centres (Scharrer, 1936; Defretin, 1955; Clark, 1956). The anterior eyes are less certainly homologous. The anterior part of the supra-oesophageal ganglion of *Nephtys* is more variable in its minute structure than the posterior part, and there are no conspicuous or constant groups of ganglion cells which can be used as landmarks. The anterior optic nerve of *Nereis* enters the brain between the two roots of the circum-oesophageal connectives; in *Nephtys* the sensory axons enter the neuropile laterally to the connective roots though immediately beside them (Fig. 2*a*). It is impossible to say how important this difference is, but the receptors themselves are in almost the same position in the two worms.

The suggestion that the eyes of the two worms are homologous is strengthened by a comparison of the experiments of Herter (1926) and Ameln (1930) on the photonegative behaviour of *Nereis diversicolor* with those on *Nephtys*. Herter showed, as a result of extirpation experiments, that *Nereis* was able to orient itself in a light beam, i.e. exhibit a phototaxis, only if the posterior eyes were intact. If they are removed the worm exhibits only a photokinesis, despite the bilateral and directional arrangement of the anterior eyes. Since the sensory data provided by the anterior eyes are theoretically sufficient to permit the worm to orientate itself in a light beam, its inability to do so is interpreted as meaning that the deficiency lies in the central nervous system. The anterior eyes of *Nephtys* are not provided with pigment cups and are not directional. The posterior eyes are both, but the worm exhibits a phototaxis only if they are close to the surface, as they are in *N. cornuta*. It is likely that the brains and photoreceptors of *Nereis* and *Nephtys* are essentially the same so far as the photonegative behaviour is concerned, save for two things: the anterior eyes of *Nereis* are directional although the sensory data they provide are not used, whereas in *Nephtys* they are not. The posterior eyes of *Nephtys*, although directional receptors, are, in most species, too deeply embedded in the ganglion to function as such.

This conclusion is based on the assumption that the structure of the supra-oesophageal ganglion is essentially the same in all species of *Nephtys*. This is not strictly true, but the arrangement of the ganglion cells in the posterior part of the brain, with which we are chiefly concerned, is the one reasonably constant feature of the ganglion. The conclusions are not proven, but are a reasonable interpretation consistent with the facts at our disposal.

Fraenkel & Gunn (1940) defined an orthokinesis as a dependence of the average

linear velocity of an animal upon the intensity of the stimulus. Within limits, the frequency of swimming of *N. cirrosa* is a linear function of the light intensity (Fig. 4). Since the duration of the swimming excursions and the speed of swimming are approximately constant whatever the light intensity (Clark, 1957), the conditions for an orthokinesis are apparently satisfied. But the worm does not swim faster in brighter light as the definition suggests, it merely swims more often, and, under natural conditions, it does not escape from the light by moving into a shadow but by burrowing out of it. Each attempt at burrowing is preceded by swimming and the first attempt may be unsuccessful, so the more frequently it swims, the sooner it will be buried. The orientation movement is therefore not orthokinetic in the proper sense of the term, but some other kind of kinetic movement. Klinokinesis, such as Ullyott (1936) described in the triclad *Dendrocoelum*, has never been observed to form part of the behaviour of *Nephtys*.

A discussion of the biological significance of this behaviour is hampered by an almost total lack of knowledge of the habits of *Nephtys*. The animals lie half buried in the sand when covered by water and may leave it temporarily to seize their food, but they must bury themselves again immediately (Clark, 1957). The stimulus initiating the last behaviour is either the exposure of the worms to light, or more likely, since they are active even in the dark if unburied, the absence of contact between the sand and the dorsum of the worm. But the time it takes to get buried depends upon the light intensity. Even so, it does not seem possible that this photonegative response can play a very important role in the normal behaviour of the worm, particularly as the photoreceptors are so primitive. Why the posterior eyes should be directional when the worm does not use them can be explained if it is assumed that the larger species have evolved from smaller ones in which much less tissue covered the receptors. They would then have been in much the same situation as *N. cornuta*.

V. SUMMARY

1. The photoreceptors found in the Nephtyidae are:

(*a*) Two pairs of vacuolated cells lying in pigment cups, with accessory cells, embedded in the posterior part of the supra-oesophageal ganglion.

(*b*) One or two cells, which may or may not be vacuolated, on either side, lying a little anterior to the ganglion.

(*c*) Undifferentiated epidermal cells surrounded by pigment granules may be photosensitive.

2. There are both morphological and behavioural grounds for concluding that the prostomial eyes of *Nephtys* are homologous with the eyes of *Nereis*, and that they are involved in the same types of behaviour.

3. The frequency with which *Nephtys* swims is, within limits, a linear function of the light intensity. Although the ganglionic eyes are directional receptors the worm does not orientate itself in a light beam; presumably the light reaching them is too diffuse. In the very small species *N. cornuta*, the eyes are close to the surface of the brain and the worm does orientate itself in a light beam.

4. Swimming is an essential prelude to burrowing, and the brighter the light the more frequently the worm swims and the sooner it is buried. Activity in light can be inhibited by stimulating receptors on the dorsal surface of the animal by contact.

This work has been carried out in the Department of Zoology in the University of Glasgow, and in the University of California during the tenure of an exchange lectureship, and at the marine stations at Plymouth, Millport and Friday Harbor, Washington. I am grateful to the heads of all these institutions for the assistance and facilities which they have so freely accorded me. I am also obliged to the Trustees of the Browne Research Fund of the Royal Society and to the National Science Foundation for grants in support of this work.

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THE BEHAVIOUR OF THE FEMALE MOSQUITO IN SELECTING WATER FOR OVIPOSITION

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INTRODUCTION

There is no doubt that mosquitoes choose certain waters in which to lay eggs; and, although we have much information about some aspects of their behaviour, we know little of what finally influences their choice. The effects of such factors as light and reflexion, vegetation, and water movement are well known, and it is clear that temperature and pH are also effective, within broad limits; much of the work on these has been done in the field, and collected accounts of it are given in the books of Marston Bates (1949) and R. C. Muirhead-Thomson (1951).

Experiments dealing with the effect of the chemical composition of water on the mosquito have frequently given a confusing picture, particularly when attempts have been made to analyse water collected from a breeding site; this is because of the large number of variables present and also because of the wide variation of conditions which may occur over a short period of time. We know little of what may be critical factors to the gravid mosquito, or about the way in which she can detect them.

In this work I have attempted to limit the number of variable factors to a minimum in order to find out whether discrimination could be made between solutions differing only in concentration. A number of simple salts, each made up in a comparable range of solutions, were offered to batches of mosquitoes, and the number of eggs laid in each concentration of a salt has been recorded. From these figures it is possible to estimate the difference in concentration which can be appreciated by the mosquitoes and to compare the behaviour towards compounds having one ion in common. These experiments form a basis for comparison with the effects of other solutions of a more complicated nature.

Some experiments were also performed in which parts of the head appendages and legs were amputated or covered to locate the position of the receptors concerned.

MATERIAL AND METHODS

Two species of Culicine mosquito were used in this work: *Culex pipiens molestus* Forskål, an autogenous and stenogamous form originally obtained from Germany in 1933 and maintained in the laboratory since then. *Aëdes* (*Stegomyia*) *aegypti* L. were originally obtained from West Africa but have been maintained in the laboratory since before 1926.

The adult *C. molestus* were maintained on sultanas and sugar solution throughout this work. *A. aegypti* were supplied with sugar solution, and females were fed on guinea-pigs. Larvae of both species were raised in tap water and fed on a mixture of dog biscuit and Bemax.

All experiments were carried out at 25° C. and at approximately 40% relative humidity. They took place in a completely darkened room in cages measuring 10 × 10 × 10 in. A period of 24 hr. was allowed from the time when test solutions were put into the cage until the egg count was made. Five-day-old females of *C. molestus* were utilized in all experiments; *A. aegypti* females were given their first blood meal when about 5 days old and used in experiments 48 hr. after this.

For *C. molestus*, where eggs are laid in the form of a raft, and where one female produces only one raft, the number of rafts per dish was taken as the measurement of choice. Eggs of *A. aegypti* were counted individually.

Two types of experiment have been carried out. In the first series I tested the mosquitoes' reaction to salt and to glucose in the water in which eggs might be laid. In the second series parts of head appendages or of legs were amputated in an attempt to locate the sense organs which enabled the female to discriminate between solutions.

Twenty-five females were counted into each cage, and totals of 375 mosquitoes were used for most experiments; where less than this number were used the fact has been noted in the results.

Twenty ml. of each solution were put into small Petri dishes, 5 cm. in diameter; nine dishes were placed on the floor of each cage in a random arrangement.

The salts used were as follows: NaCl, KCl, KNO_3, Na_2SO_4, $NaCH_3COO$, $NaHCO_3$, $MgCl_2$ and $MgSO_4$. The osmotic pressure values were calculated from concentrations and activity coefficients; where this was not possible approximate values were obtained assuming that, for dilute solutions, the gram-molecular weight in 1 l. would give an osmotic pressure of $22{\cdot}4n$ atmospheres, where n is the number of ions formed by dissociation of the compound. Solutions of glucose could be accurately made up from this formula assuming that for a unionized substance $n = 1$.

Before amputation and waxing all mosquitoes were anaesthetized with carbon dioxide. (Unoperated insects, in a control series, were found to behave quite normally about 30 min. after recovery from the anaesthetic.) Waxing of legs was carried out with a loop of platinum wire warmed electrically and dipped in a paraffin wax of low melting-point.

After amputations the mosquitoes were used in one of two ways:

(1) Given a choice between two solutions only, one known to be attractive and the other unattractive.

(2) Given a choice between a number of solutions as previously described.

For *A. aegypti*, where some form of landing place is helpful to the egg-laying female, filter-paper strips were arranged round the rims of the Petri dishes in the manner described by Wallis (1954*a*).

RESULTS

Table 1 shows the number of eggs laid in concentrations of NaCl varying from 70·5 g./l. to 0 (distilled water); these solutions were offered in groups of four. Concentrations above 8 g./l. received a very small number of rafts, but when the range was narrowed from 0 to 4·5 g./l. there was little difference in the numbers laid in each. From this it was apparent that a larger number of concentrations between 0 and 8 g./l. would have to be offered together to show where discrimination might be made.

Table 1. *Percentage numbers of rafts laid by* Culex molestus *in a decreasing series of concentrations of* NaCl, *showing the concentrations of the salt which are avoided*

No. of females per trial	Percentage no. of rafts laid	Concentration of NaCl in g./l.										Total rafts laid
		0	1·5	3·0	4·5	6·0	8·0	12·0	18·0	24·0	48·0	70·5	
450		98·0	—	—	—	—	—	—	—	0·5	1·0	0·5	206
200		74·3	—	—	—	25·0	—	0·7	0	—	—	—	141
75		43·5	—	33·5	—	23·0	0	—	—	—	—	—	30
325		35·0	27·0	14·6	23·4	—	—	—	—	—	—	—	205

In the next experiments nine dishes containing 0, 1, 2, 3, 4, 5, 6, 7 and 8 g./l. of NaCl were offered to *C. molestus* and to *A. aegypti*. Table 2 shows the percentage number of rafts and eggs laid by both species in each concentration. Both mosquitoes behaved in a similar way towards this salt and laid the larger proportion of rafts and eggs at lower salinities, but the difference in numbers of rafts or eggs per solution does not become statistically significant until the concentration reaches 0·085 M.; this value and those above it received significantly fewer eggs. From these

Table 2. *Distribution of rafts and eggs in solutions of* NaCl *by* Culex molestus *and* Aëdes aegypti

Concentrations of NaCl in g./l.	Molar concentrations of NaCl	Percentage of rafts laid by *C. molestus*	Percentage of eggs laid by *A. aegypti*
0	0	15·5	16·8
1	0·017	19·0	15·2
2	0·034	16·5	21·2
3	0·051	14·9	16·9
4	0·068	13·0	15·2
5	0·085	8·9*	7·8*
6	0·102	5·0	5·0
7	0·119	4·8	1·3
8	0·136	2·4	0·6
		100·0	100·0
	Total no. laid	248 rafts	24,348 eggs

*Significant difference at 5 % level of probability.

figures it appears that both species are able to detect a difference of 0·017 M-NaCl over the middle range of concentrations tested. This is an agreement with Wallis (1954*a*), and shows a sensitivity of an order to be associated with contact chemoreception.

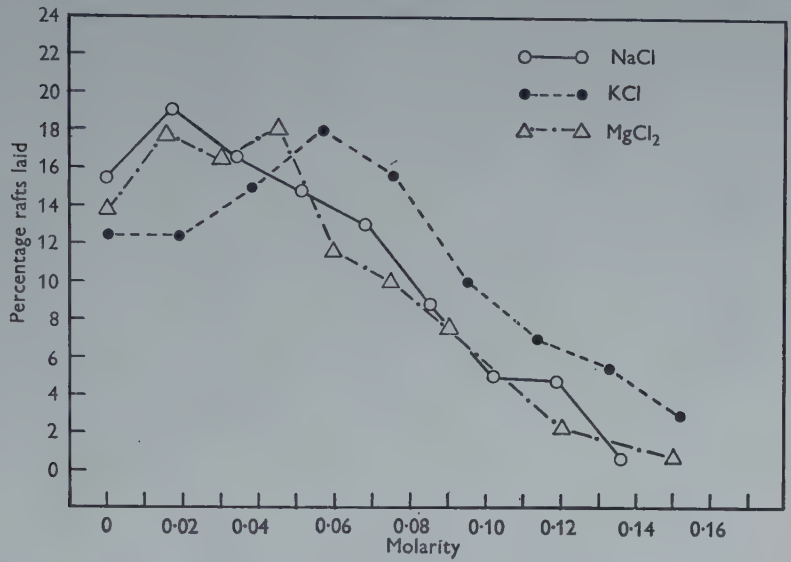

Fig. 1. Distribution of rafts in solutions of NaCl, KCl and $MgCl_2$ by *Culex molestus*.

In Fig. 1 curves have been drawn to illustrate the distribution of eggs by *C. molestus* females in solutions of KCl and $MgCl_2$; the curve obtained from the NaCl figures (Table 2) has been added. A maximum number of rafts were laid in the 0·057 M solution of KCl and in the 0·015 M solution of $MgCl_2$ with a second high number at 0·045 M. These variations are probably of little importance, since statistically there is no significant difference between the positions of the two maxima for NaCl and KCl. However, it is important to note that there is a significant difference between the numbers laid in 0·076 and 0·095 M solutions of KCl and between those laid in 0·060 and 0·075 M solutions of $MgCl_2$. The sensitivity shown here is to solutions of KCl differing by 0·019 and of $MgCl_2$ differing by 0·015 M concentrations. The behaviour towards these three chlorides is very similar, and the difference in cations present is evidently of no importance.

In the next experiment two sulphates, Na_2SO_4 and $MgSO_4$ were offered, in a similar way, to *C. molestus* females. Fig. 2 shows the pattern of distribution of rafts in each, with the NaCl curve added as a standard. Considering first the curve for Na_2SO_4, obvious discrimination is made between 0·038 and 0·057 M concentrations, showing sensitivity to a difference of 0·019 in molarity; the behaviour is comparable with that towards NaCl. The curve for $MgSO_4$ shows a random distribution of rafts over the whole range of concentrations; mosquitoes were apparently unable to discriminate between any of these solutions, or between $MgSO_4$ solutions and distilled water.

In the next group of experiments salts having a different anion were compared. Solutions of $NaCH_3COO$ and $NaHCO_3$ were offered to *C. molestus* females, and the results are shown in Fig. 3; to this the curves for Na_2SO_4 and NaCl have been added. The trend of behaviour towards all four sodium salts is very similar;

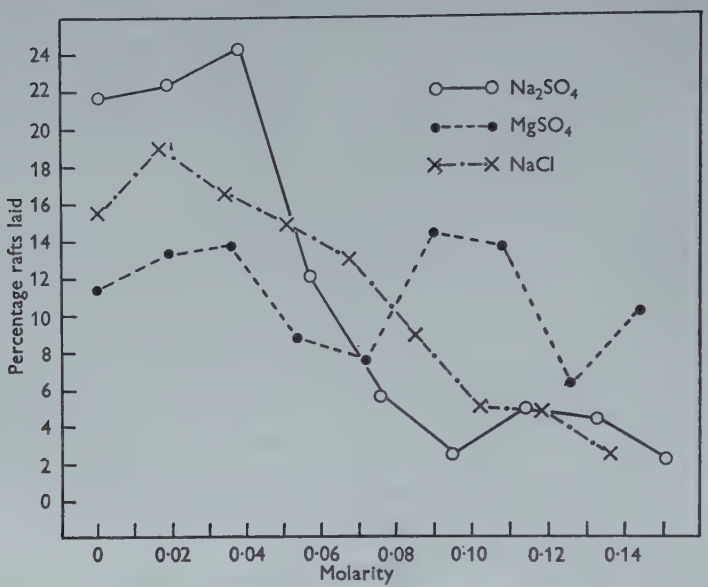

Fig. 2. Distribution of rafts in solutions of Na_2SO_4 and $MgSO_4$ and NaCl by *Culex molestus*.

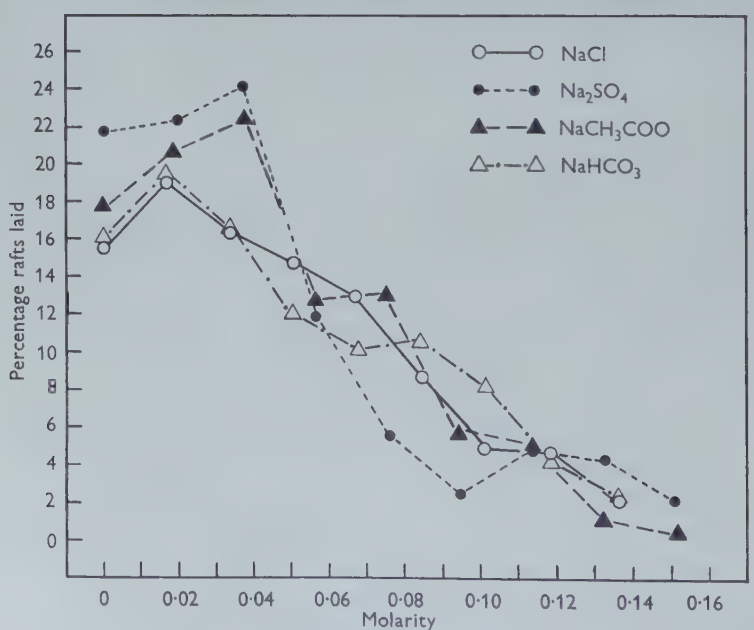

Fig. 3. Distribution of rafts in solutions of NaCl, Na_2SO_4, $NaCH_3COO$ and $NaHCO_3$ by *Culex molestus*.

however, there is some variation between the molarities of these salts at which there are significant differences in the numbers of rafts laid. For $NaCH_3COO$ the difference lies between 0·076 and 0·095 and for $NaHCO_3$ between 0·051 and 0·068 molarities. The figures for NaCl and Na_2SO_4 have been shown previously. These differences show a sensitivity to 0·019 M-sulphate and acetate and to 0·017 M-bicarbonate and chloride.

Finally, behaviour towards solutions of KNO_3 as compared with KCl and NaCl is shown in Fig. 4. The curves are essentially similar, and mosquitoes were able to discriminate between solutions of KNO_3 differing by 0·02 in molarity.

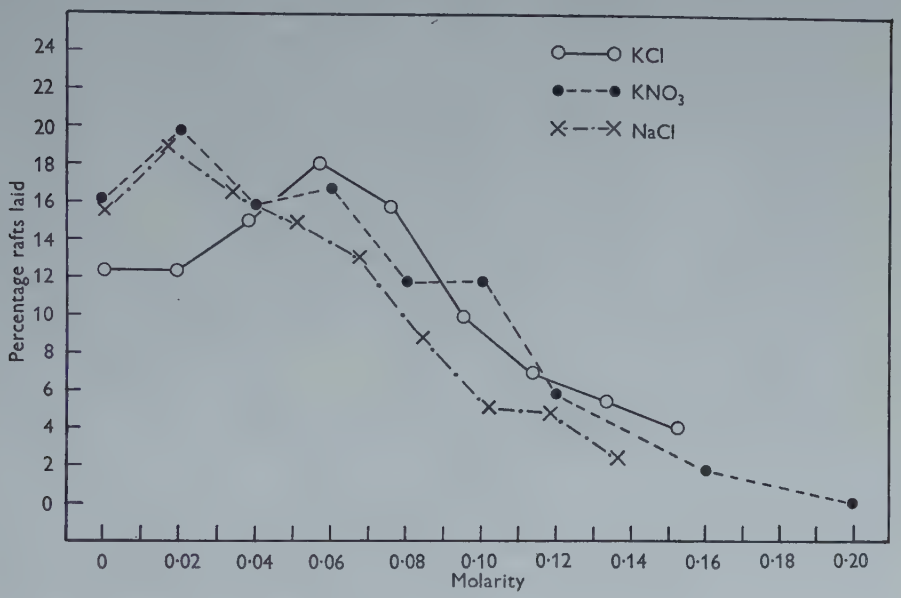

Fig. 4. Distribution of rafts in solutions of KCl, KNO_3 and NaCl by *Culex molestus*.

From the results obtained so far we know that *C. molestus* and *A. aegypti* behave similarly towards NaCl; the salt is avoided above a certain concentration which is the same for both species. This trend of behaviour was repeated by *C. molestus* when a number of different salts were offered and the presence of different anions and cations did not affect the distribution of rafts. There was, however, one exception; the distribution of rafts in the different concentrations of $MgSO_4$ chosen was obviously random. The reason for this was not apparent, since both ions had been present in other compounds and produced no unusual results; it was therefore likely to be found in some property of the compound as a whole. Calculation of the osmotic pressures given by each concentration of the salts revealed that the range of pressures covered by $MgSO_4$ was much smaller than the others; but, when these were increased (by doubling the original concentrations) so that the osmotic pressure range of $MgSO_4$ became approximately equal to that of the other six salts, the anomaly disappeared. This is shown graphically in Fig. 5; here the NaCl curve (1) illustrates the distribution of rafts both as a function of concentration (lower abscissa) and as a function of osmotic pressure (upper abscissa). Curve (2) shows

the distribution as a function of concentration of $MgSO_4$ and curve (3) shows the distribution as a function of osmotic pressure due to $MgSO_4$.

Table 3 gives figures for concentrations and osmotic pressures of NaCl as illustrated in Fig. 5. It also gives two sets of concentrations of $MgSO_4$; the first set

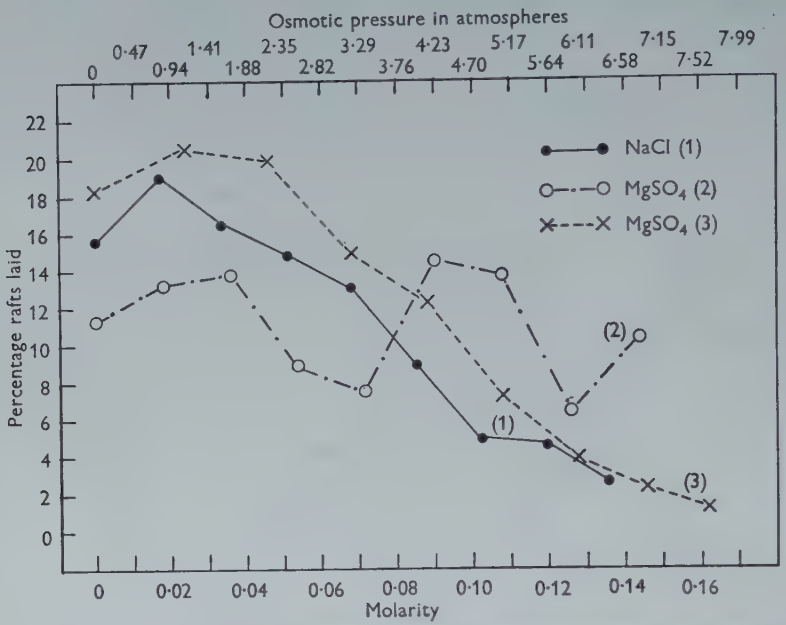

Fig. 5. Curves 1 and 2 show the distribution of rafts by *Culex molestus* as a function of molarity of NaCl and of $MgSO_4$ (lower abscissa). The scale of the upper abscissa is so arranged that curve 1 also shows the distribution as a function of the osmotic pressure of NaCl solutions. Curve 3 shows the distribution of rafts as a function of the osmotic pressure of solutions of $MgSO_4$; for $MgSO_4$ the scales of molarity and osmotic pressure are not related.

Table 3. *Percentage number of rafts laid by* Culex molestus *in solutions of* NaCl *and* $MgSO_4$. *Concentrations of the salts, with the osmotic pressure given by each, are given to illustrate the anomalous behaviour of gravid females towards* $MgSO_4$.

NaCl			$MgSO_4$				
Molar concentrations	Osmotic pressures (atm.)	Percentage no. of rafts laid	Molar concentrations	Percentage no. of rafts laid	Molar concentrations	Osmotic pressures (atm.)	Percentage no. of rafts laid
0	0	15·5	0	11·4	0	0	18·2
0·017	0·8	19·0	0·018	13·3	0·036	1·1	20·4
0·034	1·6	16·5	0·036	13·9	0·072	2·1	19·9
0·051	2·36	14·9	0·054	8·9	0·108	3·1	14·9
0·068	3·13	13·0	0·072	7·6	0·144	4·1	12·2
0·085	3·9	8·9	0·090	14·5	0·180	5·0	7·2
0·102	4·7	5·0	0·108	13·9	0·216	5·9	3·9
0·119	5·4	4·8	0·126	6·3	0·252	6·8	2·2
0·136	6·2	2·4	0·144	10·2	0·288	7·6	1·1
		100		100			100
Total no. of rafts laid		248		158			181

represents the values which produced a random distribution of rafts, and the second set the values which bring the pattern of distribution into line with that in the other salts. The osmotic pressure values of the second set of concentrations are shown and can be compared in the figure with those for NaCl.

In all the experiments so far described the compounds used have been electrolytes, and it was therefore desirable to study the effect of a series of solutions of a non-electrolyte covering approximately the same range of osmotic pressures.

Table 4. *Distribution of rafts and eggs in solutions of glucose by* Culex molestus *and* Aëdes aegypti

Concentrations of glucose in g./l.	Molar concentrations of glucose	Osmotic pressure (atm.)	Percentages of rafts laid by *C. molestus*	Percentage of eggs laid by *A. aegypti*
0	0	0	3·4	10·95
10	0·06	1·35	7·04	19·18
20	0·12	2·7	11·8	19·95
30	0·18	4·05	10·4	10·46
40	0·24	5·4	12·4	8·34
50	0·30	6·75	16·3	11·44
60	0·36	8·1	11·8	5·94
70	0·42	9·5	14·9	7·86
80	0·48	10·8	11·8	5·87
			100·0	100·0
Total no. of rafts and eggs laid			355	19,853

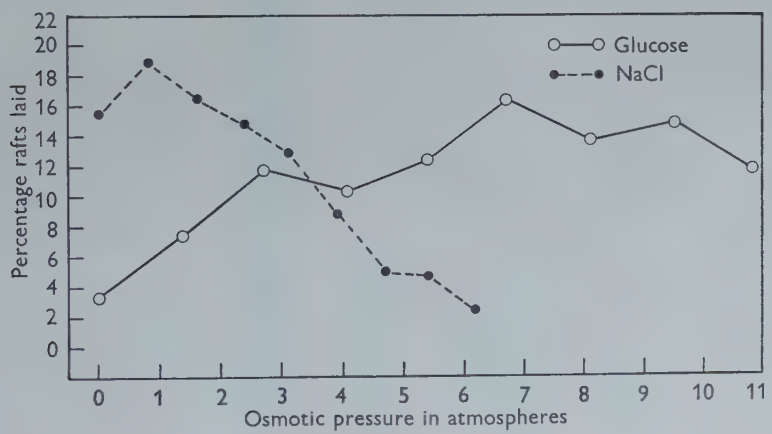

Fig. 6. Distribution of rafts in solutions of glucose and NaCl by *Culex molestus*.

Glucose solutions covering an osmotic pressure range from 0 (distilled water) to 10·8 atm. were offered to *C. molestus* and *A. aegypti* females respectively; the results of this are shown in Table 4 and in Figs. 6 and 7.

The results obtained from *C. molestus* show that comparatively few rafts were laid in distilled water or in the most dilute glucose solution. At higher concentrations, however, distribution showed no discrimination at all between osmotic

pressure values up to and including 10·8 atm. This suggests that the repellent action of higher concentrations of electrolytes was probably not due to the effect of osmotic pressure.

The curve given by *C. molestus* for the NaCl series is shown here for comparison, although this is not entirely justified, since the ranges of osmotic pressures offered differ by 4·6 atm., and it is possible that this could affect the distribution. However, such a completely different trend is shown by the two curves that further investigation is obviously required.

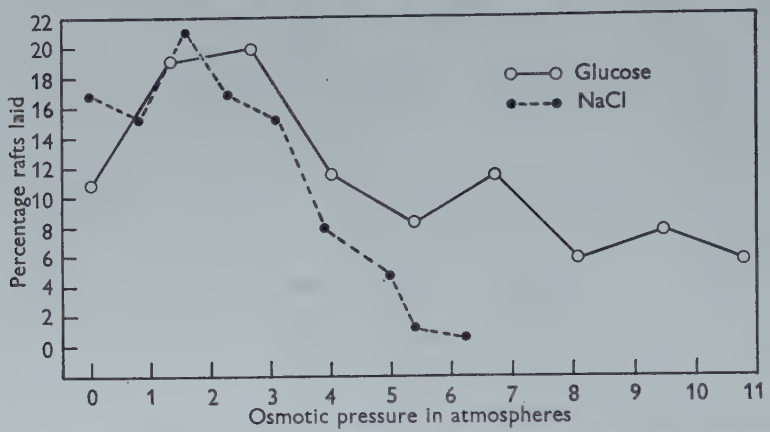

Fig. 7. Distribution of rafts in solutions of glucose and NaCl by *Aëdes aegypti*.

A. aegypti behaved rather differently towards glucose solutions; and the resulting curve shows a downward trend similar to, but not so marked as, that shown in the NaCl curve given by both species. A regression line was fitted of the number of eggs on the osmotic pressure of the solutions, and the slope was significant at the 5% level of probability; the curvature was non-significant. Therefore, it must be assumed that there was some discrimination made by *A. aegypti* in this case, between high and low concentrations of glucose.

If osmotic pressure is, by itself, responsible for selection, then isotonic solutions of glucose and NaCl should receive similar numbers of eggs if at an acceptable osmotic pressure, or should receive no eggs at all if outside the tolerated range. Solutions of NaCl and glucose were made up such that one NaCl and one glucose solution produced an osmotic pressure of 1·6 atm., and one NaCl and one glucose solution gave an osmotic pressure of 5·6 atm. These four solutions were then offered together in the same cage; twenty-five mosquitoes were used for each of six replicated trials. The results obtained are illustrated in Table 5 and by the histogram, Fig. 8. The results may be considered first from the proportion of eggs laid in the solutions of electrolyte and non-electrolyte, respectively, and secondly from the proportions laid in the solutions of high and low osmotic pressure of each substance.

In the low osmotic pressure solutions, 34·4% of the total number of eggs were laid in NaCl and 28% in the glucose. These latter figures are not significantly

different, particularly when it can be observed that in two out of six trials the greater number of eggs were laid in glucose. In the high osmotic pressure solutions, 6·2% of the total number of eggs were laid in NaCl and 31·4% in glucose. Thus, a similar proportion of eggs were laid in the high osmotic pressure glucose solution, as in the low osmotic pressure solutions of glucose and NaCl. NaCl at the higher concentration was definitely unattractive.

Table 5. *Numbers of eggs laid by* Aëdes aegypti *in two sets of isotonic solutions of* NaCl *and glucose*

No. of trials	NaCl			Glucose			Total no. of eggs laid
	1·6 (atm.)	5·4 (atm.)	Total	1·6 (atm.)	5·4 (atm.)	Total	
I	77	18	95	319	598	917	1012
II	167	27	194	450	523	973	1167
III	349	26	375	317	405	722	1097
IV	539	0	539	427	247	674	1213
V	863	130	993	387	400	787	1780
VI	990	335	1325	528	544	1072	2397
	2985	536	3521	2428	2717	5145	8666
Per-centages	34·4	6·2	40·6	28	31·4	59·4	

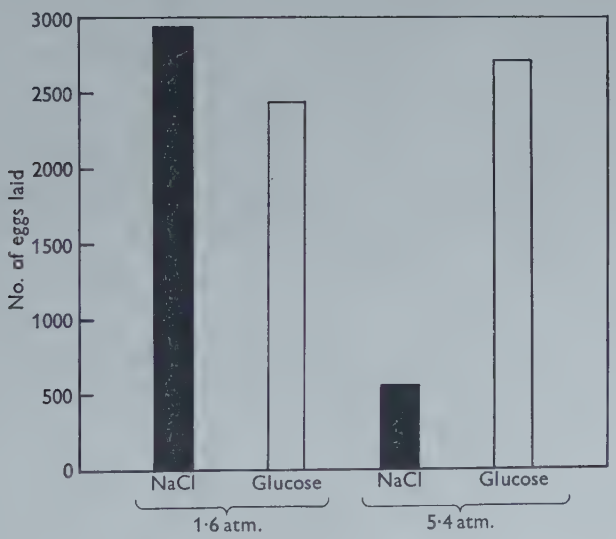

Fig. 8. Distribution of eggs in two sets of isotonic solutions of glucose and NaCl by *Aëdes aegypti*.

There can, therefore, be little doubt that there is some other influential factor than osmotic pressure involved in the discrimination that is observed.

Throughout the previous series of experiments observations on the sequence of behaviour of mosquitoes before and during oviposition were made. These were

possible using a dimmed electric torch which did not apparently disturb the mosquitoes and gave a reasonably detailed view of activity.

Prior to oviposition, *C. molestus* females are fairly quiescent and distributed over all the sides of the cage. Some time after the introduction of dishes of solutions a few females leave the sides and fly backwards and forwards across the dish. They do not seem to touch the surface during initial flights, but eventually settle for varying lengths of time. If the solution is favourable, they may remain and carry through the egg-laying procedure or they may leave the dish and continue trials with others, possibly returning to the original dish and finally laying eggs there. In any case, contact with one or more solutions is definitely made before egg-laying takes place; this observation was borne out by some experiments in which the surfaces of solutions were marked with coloured wax powders which could be traced on the mosquitoes after oviposition.

Initially, the contact appeared to be made with the pro- and mesothoracic legs, as the meta-legs are held characteristically curved upwards. Before egg-laying actually commences the meta-legs are lowered on to the surface so that the tarsi lie horizontally along it, and the distal parts of the tibiae are in contact with it. The distal tarsi are then moved towards each other until the two legs form a pen into which the eggs are deposited and formed into a raft. The use of the meta-legs during oviposition made amputation experiments on this species inadvisable.

If a female rested for some time on a solution, it was observed that the proboscis was put into the liquid and drinking appeared to take place. This was a very normal procedure with molestus, and it was thought that the mouthparts, or indeed some part of the foregut, might assist in the mechanism of discrimination. This possibility was investigated by amputation experiments.

There was also the possibility that the tip of the abdomen, or some part of its ventral surface, might bear sensilla of the chemosensory type, but these are difficult areas to cover without also damaging the structures used in laying eggs.

Location of areas concerned with the choice between solutions for egg-laying

To discover if drinking the solution assisted the female mosquito in choosing a suitable water, seventy-five females with the proboscis amputated and the remaining stump waxed over were offered a choice between distilled water and 8 g./l. of NaCl. Forty-two rafts were laid in distilled water and none in NaCl; the controls behaved similarly.

A number of experiments were performed in which the whole series, from 0 to 8 g. l. of NaCl were offered to *C. molestus* females with: (1) proboscis amputated, (2) palps amputated, (3) both proboscis and palps amputated. In none of these was the result significantly different from the unoperated controls. Similarly, amputation of the antennae of 100 females gave no evidence that these assisted the choice of a solution.

It has been shown that the ovipositors of *Nemeritus canescens* respond to contact chemical stimuli (Dethier, 1947), and it was thought possible that this might be the situation with mosquitoes. Several attempts were made to isolate areas all round

the ovipositor from contact with the solution, but as a result of these operations no eggs were laid. A successful method of preventing the ovipositor from touching the surface of a solution has been described by Wallis (1954*a*). He found that ten species of mosquitoes were able to detect distilled water from a 3 per cent saline solution with the ovipositor isolated from contact with either.

Exclusion of sensory areas on the legs was carried out by covering them with a wax film in the manner previously described or by the amputation of parts. The initial experiments in this series were performed on *C. molestus* females; but, as had been mentioned, interference with the metathoracic legs, either by stiffening them with wax or by their removal, involved a mechanical disturbance of the egg-laying procedure. Mortality rates were very high and the results difficult to assess because of the way in which eggs were deposited; rafts were very poorly formed or not formed at all; it appeared that a large number of females drowned after laying only a few eggs. Therefore, experiments were transferred to *A. aegypti*.

Table 6. *Distribution of eggs in distilled water and* 8 g./l. *of* NaCl *by* Aëdes aegypti *females with all legs amputated to the tibio-femoral articulation*

No. of trials	Tarsi and tibiae amputated			Control unoperated		
	No. of eggs laid in			No. of eggs laid in		
	Distilled water	NaCl (8 g./l.)	Total	Distilled water	NaCl (8 g./l.)	Total
I	81	40	121	447	1	448
II	83	114	197	1170	108	1278
III	126	39	165	1052	2	1054
IV	67	107	174	978	222	1200
V	74	129	203	719	0	719
	431	429	860	4366	333	4699
Percentages	49·8	50·2		92·9	7·1	

Table 6 shows the numbers of eggs obtained from five groups of fifteen *A. aegypti* females, with six legs amputated at the tibio-femoral joints; the cut surfaces were waxed over. The results from experimental insects are compared with a control series.

No discrimination was made by operated insects between the saline solution and the distilled water, indicating that the sense organs responsible for selection are situated below the tibio-femoral articulation.

In the following experiment (Table 7) an attempt was made to show whether the presence of the tibiae increased the ability of *A. aegypti* females to discriminate between an 8 g./l. NaCl solution and distilled water. All tarsal segments were amputated from the legs of five groups of fifteen females, the amputation being done just above the tibio-tarsal joint, and the cut surface waxed over. Experimental results are compared with those from unoperated controls.

The results from this are not conclusive, but it appears from the percentages of

the total numbers of eggs obtained that retention of the tibiae of all legs has enabled some discrimination to take place. Although analysis of individual results shows that in three out of five trials a greater number of eggs was laid in the saline solution, this is of doubtful significance because the numbers of eggs laid in a given dish are only roughly representative of the numbers of females which have oviposited there. Where such high mortality rates occur a larger number of trials should be carried out. The results obtained do not agree completely with those of Wallis (1954*a*), who worked with ten different species, including *A. aegypti*, and found that ten females of each species, with all tarsal segments absent, were unable to discriminate between a 3% NaCl solution and distilled water.

Table 7. *Distribution of eggs in distilled water and* 8 g./l. *of* NaCl *by* Aëdes aegypti *females with all legs amputated at the tibio-tarsal articulation*

No. of trials	Tarsal segments amputated			Control unoperated		
	No. of eggs laid in			No. of eggs laid in		
	Distilled water	NaCl (8 g./l.)	Total	Distilled water	NaCl (8 g./l.)	Total
I	17	292	309	447	1	448
II	445	111	556	1170	108	1278
III	167	185	352	1052	2	1054
IV	159	217	376	719	0	719
V	694	217	911	907	187	1094
	1482	1022	2504	4295	298	4593
Percentages	59·2	40·8		93·5	6·5	

Wallis (1954*a*) also showed, by serial amputation of the tarsi of different legs, and by amputation of the individual tarsal segments, that contact chemoreceptors are distributed on all segments of all the tarsi.

DISCUSSION

Other investigators, working with many sorts of mosquitoes, in the field and in the laboratory, have shown that gravid females do select certain waters for oviposition. Here, *C. molestus* and *A. aegypti* have been shown to discriminate between simple salt solutions in which the number of influential factors has been reduced to a minimum. Thus, on the assumption that all other effects have been eliminated, or standardized, the interpretation of results is restricted to a consideration of the properties of the salts themselves.

Of the physical properties of such solutions the following factors were investigated:

(1) *Vapour pressure*. This factor alone was considered unlikely to be responsible for the discrimination shown because differences in vapour pressure over such a range of concentrations would be very slight indeed, also, because these differences would be confined to a very thin layer above the surface of the solution.

(2) *Surface tension.* Differences in surface tension between solutions offered are very small indeed.

(3) pH. The pH of all solutions offered was very close to neutral. Experiments in which solutions varying in pH were offered to *C. molestus* females indicated that any values between pH 5 and 8 were equally acceptable.

A comparison of behaviour towards salts having one ion in common did not reveal any one to be more or less attractive than the other; in fact, the close similarity of results indicated a correlation between the reaction of the ovipositing female and some property common to all these compounds. The fact that $MgSO_4$ did not produce a typical pattern of behaviour, until the osmotic pressures of all its concentrations were made equal to those of the other solutions, suggested that this factor might be a critical one. This appears to be disproved by the behaviour towards glucose solutions covering a similar osmotic pressure range; however, there is a possibility that the effect of glucose on ovipository behaviour could be masked by its effect on the feeding responses, particularly in the case of *C. molestus*. This point could be clarified by offering a series of solutions of some substance unlikely to be acceptable as a food.

It is interesting to note here that Frings (1946), working on *Periplaneta americana*, compared series of ions for stimulative efficiency and found that magnesium ranked low in stimulating power, the order of cations shown being also that of ionic mobilities. In an empirical arrangement of anions, chloride and sulphate were rated about equal in efficiency. Also, Hodgson, in a determination of reaction thresholds for *Laccophilus maculosus* Germ., found that magnesium, calcium, copper, ferric and barium chlorides failed to produce a typical response even at concentrations above the ordinary physiological range. This was not due to high valency or molecular weight as such.

During the course of experiments with *C. molestus*, a tendency to drink from the solution offered for egg-laying was observed. Wallis (1954*a*) describes drinking by *Anopheles quadrimaculatus* but isolates this as a function distinct from oviposition. It has been proved for *C. molestus* that drinking is not a necessary feature of pre-ovipositional behaviour, but the fact that it is often initiated, particularly in low saline concentrations, might be of assistance in future experiments where it is desired to make use of some more critical response than oviposition.

Experiments to locate the position of contact chemoreceptors have confirmed the findings of Frings & Hamrum (1950) and Wallis (1954*a*) that these are situated on the legs. This goes far towards explaining a number of observations made on the behaviour of certain species before and during oviposition (Russell & Rao, 1942; Kennedy, 1941; d'Arbrera, 1944; Buxton & Leeson, 1948). It has been difficult to envisage a type of response which would explain both the behaviour of culicines, which maintain contact with the surface throughout oviposition, and that of certain anophelines, which are apparently able to oviposit while hovering above the surface. It is possible to suggest that some brief contact, made at such speed that it is not always apparent to an observer, is made during exploratory flights. The stimulus obtained in this way must be sufficient to initiate oviposition, or to promote a search

for a more favourable solution; however, further experimentation is required to confirm this. Positive identification of the end-organ concerned is an essential requirement for any investigations of this type.

It has been shown quite definitely by Wallis (1954*a*) and in this work that the sense organs concerned in the selection of water for oviposition are situated on the tarsi of all legs; results here have also indicated that the distribution of the receptors may extend to the tibiae.

SUMMARY

1. Egg-laying *Culex molestus* and *Aëdes aegypti* were able to discriminate between solutions of NaCl ranging from 0 (distilled water) to 0·136 M. Significantly fewer eggs were laid in solutions above 0·085 M.

2. Similar series of solutions of KCl, $MgCl_2$ and Na_2SO_4 showed a similar distribution of eggs, but mosquitoes were apparently unable to distinguish between $MgSO_4$ solutions below 0·144 M.

3. Results from all these salts were related to the osmotic pressures produced, but experiments in which isotonic solutions of glucose and NaCl were offered simultaneously showed that osmotic pressure was not a critical factor.

4. Experiments were carried out to locate the sensory areas responsible for discrimination. The possibility that drinking might be associated with the choice of a solution for egg-laying was investigated by removing the proboscis; operated insects were still able to detect differences in concentration. Covering or removing various regions of the legs revealed that the chemoreceptors concerned were distributed on all the tarsi; indications that they may also be found on the tibiae were obtained.

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THE SENSITIVITY OF THE PEDAL GANGLION OF THE SLUG TO OSMOTIC PRESSURE CHANGES

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INTRODUCTION

The blood in the living slug can have a wide range of concentrations, depending on whether the animal is in a dehydrated or hydrated condition. In an earlier paper by Hughes & Kerkut (1956) it was shown that one could make a quantitative study of the spontaneous activity of a single unit in the isolated pedal ganglion of the slug. It was found that this preparation was sensitive to changes in the concentration of the bathing solution; the cells were more active in dilute Locke solution and less active in concentrated Locke solutions. Furthermore, the preparation was sensitive to experimental changes of concentration one-twentieth those that can occur in the blood of the living animal. However, it was not clear whether this effect was due to the osmotic pressure of the solution or whether it was due to the change in the ionic concentration, i.e. the effective concentration of Na^+, K^+, Ca^{2+}, Mg^{2+}. The experiments described in this paper will show the effect of solutions of identical ionic composition but differing osmotic pressure on the activity of the pedal ganglion cells.

In the earlier account the changes found to occur in the isolated pedal ganglion following concentration or dilution of the Locke solution were postulated as being similar to those that would occur in the intact animal following dilution or concentration of the blood, but there was a major difficulty to this interpretation. We had studied the effect of sudden changes in the concentration of the bathing medium, but the changes that take place in blood concentration following hydration or dehydration of the slug are gradual. It was therefore necessary to determine the effect of similar gradual changes on the activity of the isolated pedal ganglion before one could apply these results to the living animal.

METHOD

The apparatus used in these experiments is similar to that described by Hughes & Kerkut (1956). The slug brain was dissected and immersed in earthed Locke solution. A fine tungsten wire electrode was inserted into the ganglion and the impulses led to a Leak amplifier and then to a Cossor double-beam oscilloscope. The impulses were monitored on the oscilloscope and then counted on a dekatron scaler (Kerkut, 1955). Identical results were obtained from both *Agriolimax reticulus* and *Arion ater*.

RESULTS

(1) *Effects of solutions of differing osmotic pressure on the pedal ganglion*

The effect of solutions of identical ionic composition but different osmotic pressure is shown in Fig. 1. Locke solution was diluted to half strength (0·5 Locke) and glucose added to it; 17·71 g. of glucose were added to each litre of 0·5 Locke, and this solution was referred to as 0·5 G. The preparation showed an activity of approximately 10 impulses/half-minute in 0·7 Locke, and the mechanical act of changing the solution as illustrated by a change from 0·7 Locke to 0·7 Locke had no effect on the activity.

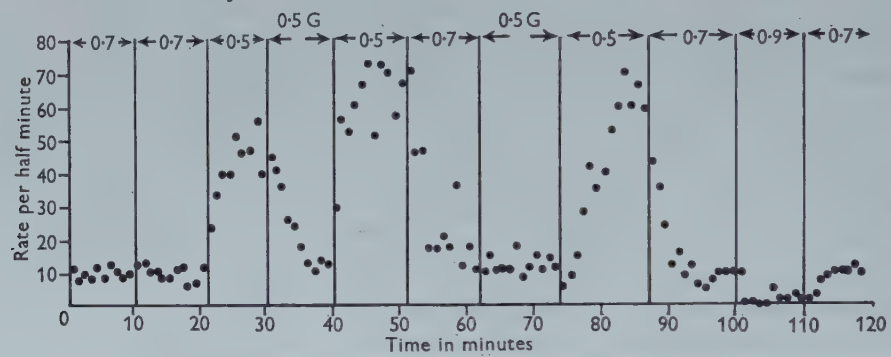

Fig. 1. The effect of solutions of the same ionic concentration but different osmotic pressure on the activity of the pedal ganglion. The solution of 0·5 G contained 0·5 Locke plus 17·71 g. glucose/l. It will be seen that this behaved much like 0·7 Locke.

When the solution was changed from 0·7 to 0·5 Locke there was a marked increase in the activity from 10 to 45 impulses/half minute. When the bathing solution was now changed from 0·5 Locke to 0·5 Locke plus glucose (0·5 G), the activity fell from 45 to 14 impulses/half minute. The preparation regained its activity to 0·5 Locke and fell off again in 0·7 Locke. It is interesting to note that there was little or no change in the activity of the preparation when the solution was changed from 0·7 Locke to 0·5 G. Thus 0·5 G behaved like 0·7 Locke, even though it had a different ionic concentration.

The glucose did not have any noticeable deleterious effect on the preparation, since after treatment with 0·5 G the ganglia behaved quite normally to subsequent treatment with 0·5, 0·7 and 0·9 Locke solutions.

Similar results were obtained by adding mannitol to Locke solution. Results of some of these experiments are shown in Figs. 2 and 3. Fig. 2 shows the effect of adding different concentrations of mannitol to 0·5 Locke. The preparation showed 13 impulses/half minute in 0·5 Locke + 17·7 g. mannitol/l.; 4 impulses/half minute in 0·5 Locke + 35·42 g. mannitol/l.; 27 impulses/half minute in 0·5 Locke.

The effect of mannitol is further demonstrated in Fig. 3. The rate of activity was constant in 0·5 Locke and greatly increased if the solution was changed to 0·25 Locke. If, however, the preparation was placed in solutions of 0·5 Locke which had various concentrations of mannitol added, then a series of curves was obtained

which showed that the activity was highest in those solutions that contained the least mannitol, and lowest in those solutions that contained the most mannitol. The rate of activity was thus inversely proportional to the concentration of mannitol

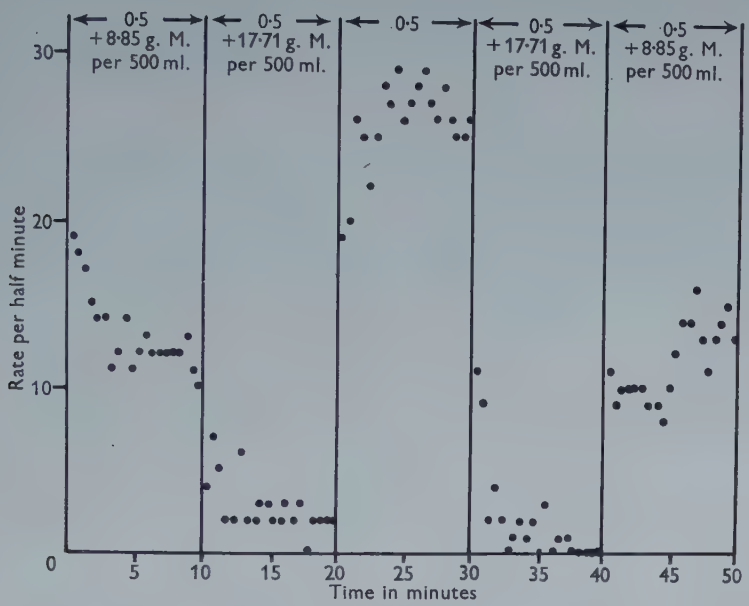

Fig. 2. Effect of solutions containing mannitol on the rate of activity of the pedal ganglia. The activity is highest in solutions containing low mannitol concentrations.

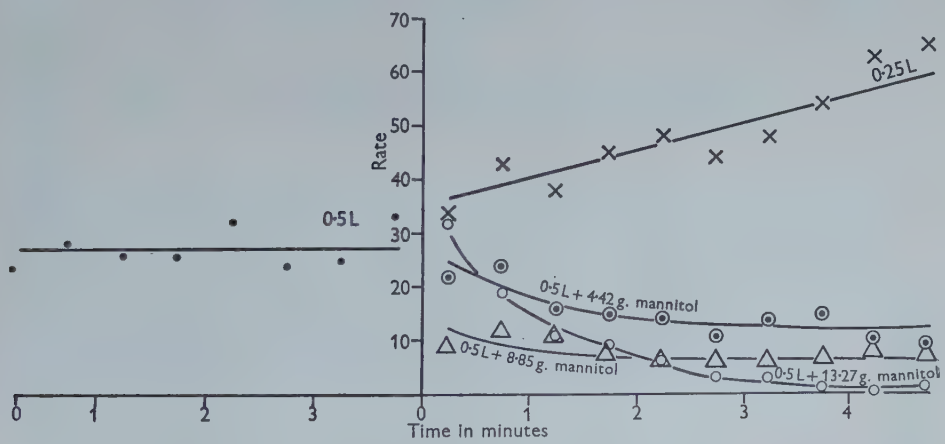

Fig. 3. Effect of solutions containing mannitol on the rate of activity of the pedal ganglion. The activity is approximately inversely proportional to the mannitol concentration.

in the solution, even though all these solutions had the same ionic composition. These experiments indicate that the pedal ganglion is probably responding to changes in the osmotic pressure of the solution and not to the total ionic concentration.

(2) *Gradual changes in the concentration of the bathing medium*

As mentioned in the introduction, the changes in blood concentration following hydration or dehydration of the living animal are probably gradual. It was therefore necessary to decide whether the pedal ganglion in the intact animal would show changes in activity following gradual changes in the concentration of the bathing solution.

We were lucky in our investigation of this effect in that one of the first preparations so studied showed peculiar properties. This preparation was active in 0·7 Locke and more dilute solutions, but was totally inactive in 0·8 Locke and more concentrated solutions. We therefore immersed the ganglion in 0·8 Locke and gradually diluted the medium. A burette containing dilute Locke or distilled water was placed to one side of the bath and the rate of flow controlled by the burette tap.

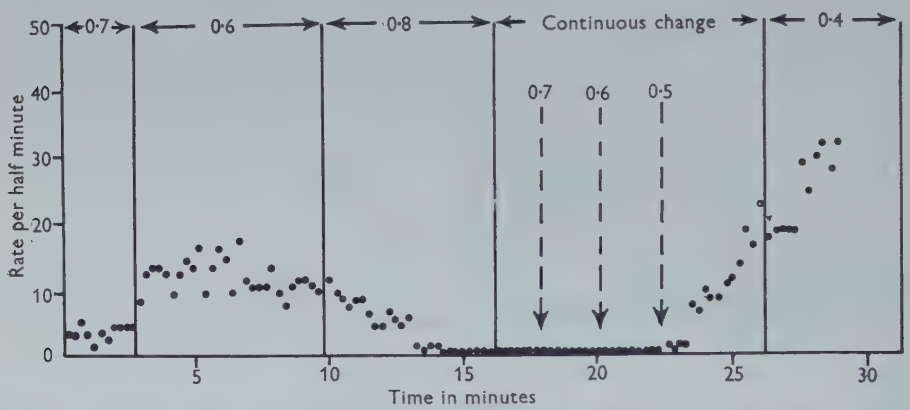

Fig. 4. Effect of gradual dilution from 0·8 Locke to 0·4 Locke over 10 min. The activity increased as the solution became more dilute.

By knowing the initial volume of fluid in the bath and the volume of fluid added, one could calculate the dilution. The preparation was protected from the burette by a series of baffles, and the solution was thoroughly mixed by an aerator. The first change took 10 min. to complete, the change being from 0·8 Locke to 0·4 Locke. It will be seen from Fig. 4 that the activity of the preparation increased after dilution. Fig. 5 shows the effect of a slower dilution, the change from 0·8 Locke to 0·4 Locke taking 50 min. The times taken for the solution to reach the various dilutions intermediate between 0·8 Locke and 0·4 Locke are shown on the graph.

The effect of smaller gradients (i.e. smaller dilutions over a longer time) is shown in Fig. 6. In Fig. 6 the effect of a gradual change from 0·7 Locke to 0·6 Locke over 43 min. is shown. Here we have plotted the running mean of the activity against time, this method having the advantage of smoothing out the curve and showing the general trend more clearly. Though the record shows some perturbations there is a gradual increase in the level of activity.

Fig. 7 shows the effect of diluting the bathing medium from 0·5 Locke to 0·4 Locke, the change taking place over 44 min. The effect of gradual dilution is to increase the activity of the preparation, though it will be noted that there was a certain degree of adaptation, i.e. after some time the activity slowly fell off. Even so the activity at the end of the dilution was clearly greater than that observed at the beginning of the dilution.

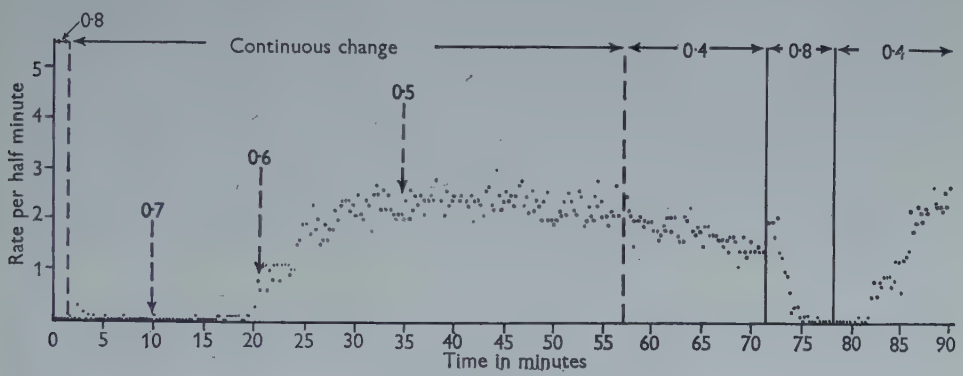

Fig. 5. Effect of gradual dilution from 0·8 Locke to 0·4 Locke over 50 min. The activity in general is increased as the solution becomes more dilute.

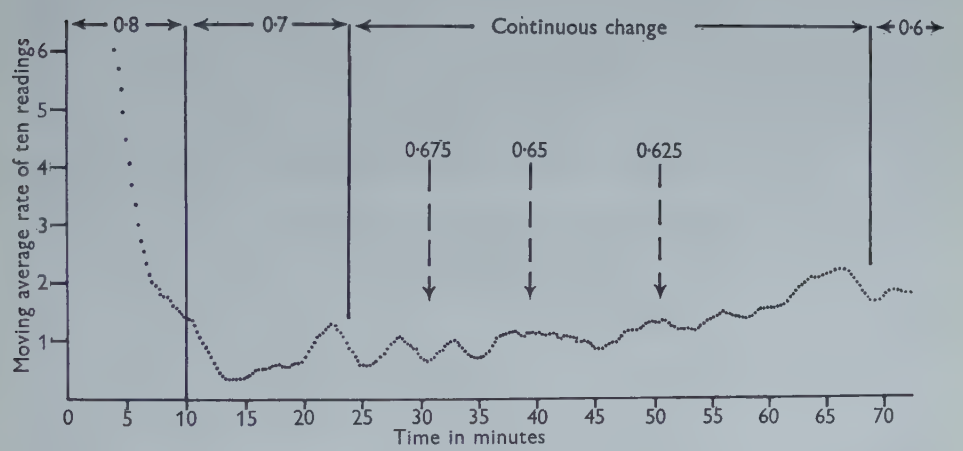

Fig. 6. Effect of gradual dilution from 0·7 Locke to 0·6 Locke over 43 min. The running mean of the rate is plotted in order to show the general trend more clearly. The rate increases as the solution becomes diluted.

We chose to examine the effect of gradual changes by diluting rather than by concentrating the medium, since the normal trend of a preparation remaining in a given solution is gradually to diminish its rate of activity. Thus we might have observed a slow decrease in activity following the gradual concentration of the solution, but would have been unable to decide whether this was due to ageing of the preparation or the effect of the more concentrated solution.

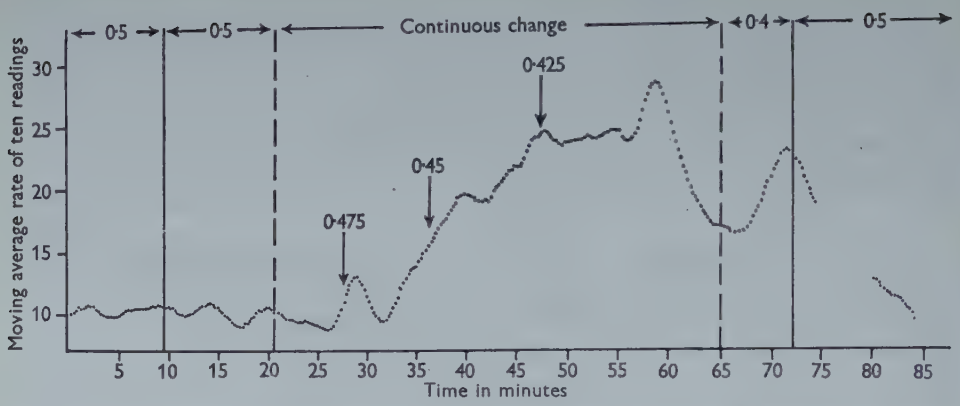

Fig. 7. Effect of gradual dilution from 0·5 Locke to 0·4 Locke over 44 min. Though there is some irregularity in the rate of activity the general trend is for the rate of activity to increase following dilution.

DISCUSSION

The experiments described in the previous section raise three points: (*a*) the relative importance of ionic and osmotic changes; (*b*) whether the results from the *in vitro* experiments can be applied to the intact animal; (*c*) the agreement of the present results with those previously described for other osmoreceptors.

One can see from the experimental results that the important factor in changing the solution is the change in osmotic pressure of the solution. Diluting the solution and so lowering its osmotic pressure makes the pedal ganglion more active, whilst concentrating the solution and so raising its osmotic pressure makes the preparation less active. The effects of dilute and concentrated solutions can be obtained by using solutions containing the same ionic concentration but differing amounts of glucose or mannitol.

This is not to say that the ionic concentrations are of no importance to the animal, though they are most clearly shown when the relative concentration of different ions such as calcium or magnesium is changed. These experiments are for the most part artificial, since such conditions do not often occur in nature. However, Lustig, Ernst & Reuss (1937) have shown that the hibernating snail has a higher concentration of magnesium in its blood than has the active animal. Experiments on the effects of changes in ionic balance will be presented in a later paper. In the living animal the effect of hydration or dehydration is presumably to alter the total concentration of the ions and thus the effective osmotic pressure of the blood; there is no evidence that the relative concentrations of ions are altered. Thus we may conclude that the important factor in the intact animal is the effective osmotic pressure of the blood.

It is now necessary to show that the osmotic pressure changes in our experiments on the isolated pedal ganglia are similar to the changes that can occur in the intact animal. In the live desiccated slug, the blood is isotonic with a 1·4 Locke solution. In a live hydrated animal the blood is isotonic with a 0·4 Locke solution. The isolated pedal ganglion is sensitive to a sudden change from 0·7 Locke to one of

0·65 Locke, i.e. a change one-twentieth the intensity of that which can occur in the living animal. However, this comparison is not strictly valid, since in the living animal the changes are probably gradual. Thus we must consider the rate of change and not only the absolute change itself.

Experiments show that a slug can increase its body weight by 30% in 1 hr. hydration. The increase in weight is mostly due to the uptake of water into the blood, the blood being the most sensitive of the tissues to hydration or desiccation (Pusswald, 1948). If we take the standard osmotic pressure of slug's blood as isotonic with 0·7 Locke, then a 30% dilution of the blood would make it approximately isotonic with 0·5 Locke. The change in the slug's blood concentration would be 0·7–0·5 over 1 hr. We have shown that the isolated preparation is sensitive to a slower rate of change than this, i.e. 0·7 Locke to 0·6 Locke in 43 min. Thus the experimental conditions are if anything less vigorous than those that can occur in nature.

In studying the effect of sudden changes of concentration of the bathing solution on the activity of the pedal ganglion, Hughes & Kerkut (1956) found that some preparations showed adaptation. That is, after the solution had been diluted the activity of the preparation increased, reached a peak after some 20 min. and then fell off. This adaptation might be of considerable importance in the living animal. Thus the normal animal in the rain would have water slowly entering its body and diluting its blood. If the rate of dilution was equal to the rate of adaptation, the pedal ganglion would be unaffected by the change in the blood's concentration. In fact, experiments on the effect of gradual changes in concentration show that unless the rate of hydration or dehydration is extremely low there is a detectable effect on the activity of the preparation. Though some adaptation may occur following dilution, all preparations show that the activity after dilution is greater than the initial activity.

The reactions of osmoreceptors have been studied in other animals. Verney (1947) showed that the mammalian receptors were a series of nerve cells lying in the hypothalamus. If the blood was diluted, these cells became vacuolated (Jewell, 1953), and it is thought that in some way they affect the secretion of the anti-diuretic hormone.

Verney showed that the mammalian osmoreceptors were sensitive to a 2% change in the osmotic pressure of the blood. If we take blood as being isotonic with Locke solution, then the mammalian receptor is sensitive to a sudden change of 2% Locke, whilst the slug pedal ganglia are sensitive to a sudden change of 5% Locke. On the other hand, the slug can detect a change of 10% Locke taking place over 43 min. i.e. a change of the order of 1% in 4 min.

C. von Euler (1953) showed that the injection of 2% NaCl (Locke is approximately 1% NaCl) into the hypothalamic region of a cat led to a slow potential (recorded between the supraoptic region of the hypothalamus and the frontal air sinus) lasting approximately half a minute. A similar potential was evoked by injecting 10% glucose solution, whilst injection of tap water brought about a slow potential of opposite sign.

There are certain references in the literature to the sensitivity of nerve cells to osmotic changes. Libet & Gerard (1938, 1939), studying the waves of electrical activity in the isolated frog brain, found that the potentials were in some cases sensitive to changes in the osmotic pressure of the solution. Addition of glucose increased the amplitude of the waves in some cases from 45 to 120 μV. The wavelength was slightly increased from 0·2 to 0·25 sec., and thus the frequency was slightly lower. This then agrees with our findings in the slug that increasing the osmotic pressure results in a lower frequency of discharge, though Libet & Gerard were here studying slow waves and not the activity of single units.

Fatt & Katz (1952) found a different response to osmotic change. They studied the spontaneous subthreshold potentials in the motor end-plate of the frog. These were affected by changes in the osmotic pressure of the bathing solution. A 50% increase in the osmotic pressure (by addition of sucrose) led to an increase in the frequency of the potentials from 2 to 90/sec. Further experiments showed that raising the osmotic pressure 30% increased the rate from 15 to 150/sec., whilst decreasing the osmotic pressure by 50% reduced the frequency from 28 to 0·9/sec. Thus in the frog subthreshold end-plate potentials, the frequency is *increased* by *increasing* the osmotic pressure of the solution, whereas in the slug the frequency of the potentials is *increased* by *decreasing* the osmotic pressure of the solution.

Alanis & Matthews (1952) showed that the nerve cell bodies in the ventral horn of the frog spinal cord were sensitive to mechanical pressure. Pressure on the cell body tended to depolarize the membrane and facilitate the development of action potentials. We have noticed in the slug that pressure on the ganglion tends to elicit a higher frequency of action potentials.

If the neurone behaves like a single osmometer it should swell in dilute solutions and so distend the membrane. This would have an effect similar to that of mechanical pressure on the membrane and so facilitate the development of action potentials. On the other hand, it would also be necessary to postulate some means of adaptation, since animal cells, unlike plant cells, are not surrounded by a thick cellulose sheath which prevents them from swelling. The pedal ganglion is surrounded by a collagen sheath, but this appears to play little part in the sensitivity of the preparation to osmotic changes, since the preparation is still sensitive to osmotic changes even if the sheath is slashed. It is more probable that the nerve membrane either alters its permeability to the substances outside the nerve, or that the membrane in some as yet unknown way changes its sensitivity.

SUMMARY

1. The effects of different dilutions of Locke solution on the electrical activity of the isolated pedal ganglion of the slug can be reproduced by adding different concentrations of glucose or mannitol to a given concentration of Locke.

2. This indicates that certain cells in the pedal ganglion are sensitive to the osmotic pressure of the solution and not its ionic concentration.

3. The preparation is sensitive to slow changes in the concentration of the bathing medium. The cells increased their activity when the bathing solution was slowly changed from 0·7 Locke to 0·6 Locke, the change taking 43 min. This corresponds approximately to a change of 1% of the body fluid concentration over 4 min. Such rates of change are found in the normal intact animal.

4. The sensitivity of the preparation compares well with that of the mammalian osmoreceptors.

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BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF THE PURIFIED TOXIN OF *WALTERINNESIA AEGYPTEA* 'THE EGYPTIAN BLACK SNAKE'

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INTRODUCTION

Walterinnesia aegyptea or 'The Black Snake' which lives in the Sinai Desert near Suez is extremely poisonous. The snake was named after Walter Innes (1923), who was the first to discover the presence of the snake in the Eastern Egyptian Desert. Many fatal attacks of this snake upon camping soldiers and local inhabitants have been reported. Anderson (1925) made a valuable zoological study of the snake. Curckill (1929) described two cases of death occurring as a result of the bite of this snake. Death occurred from 6 to 24 hr. after the bite. Biochemical and physiological studies of venoms from different poisonous animals living in Egypt have been reported by many investigators, e.g. Wilson (1921), Shousha (1928), Hassan and Mohammed (1940) and Karimi (1955). Physiological effects of the different snake venoms were reviewed by Essex, (1945).

In this work a technique for preparing the toxin of *W. aegyptea* in a crystalline form is described. The minimum lethal dose of the toxin has been determined. Biochemical and physiological studies are reported.

PREPARATION

(1) *First method.* The snake is allowed to bite a rubber membrane covering a small beaker. The volume of the venom thus collected is about 0·7 ml. It is a viscous yellowish liquid. It is diluted 100 times with distilled water, then clarified by addition of about 1 g. aluminium sulphate crystals followed by 5 ml. of lime water. Pure acetone is added gradually until a heavy flocculent precipitate is obtained. This is then centrifuged and is washed three times with acetone, then with ether. Finally the precipitate obtained is dried under vacuum. The residue thus obtained is a white amorphous substance. The venom can be obtained in a crystalline form if rewashed with ether, centrifuged, then dried under reduced pressure over phosphorous pentoxide at 30° C.

(2) *Second method.* The venom collected after one bite is diluted 100 times with 0·1 N-HCl, then neutralized to pH 7 by addition of N-NaOH. The solution is saturated with picric acid crystals, and then allowed to stand for 24 hr. The toxin thus precipitated is centrifuged, then repeatedly extracted with 80% acetone in water (v/v) until no further picrate is recovered. A few drops of conc. HCl are

added to the toxin, followed by excess of pure acetone. The precipitated toxin hydrochloride thus obtained is centrifuged, then washed successively with acetone and ether. Finally, the crystalline salt of the toxin is obtained by drying the precipitate in vacuum over phosphorous pentoxide.

The yield of one bite (about 0·7 ml.) for either method is 35–50 mg. of fine crystalline toxin.

BIOCHEMICAL STUDY

The purified crystalline toxin dissolves readily in distilled water to give a colourless but frothy solution. It is found to be slightly alkaline (pH = 7·4). The toxin can be precipitated from the aqueous solution by alcohol, acetone or ether. The dried toxin leaves no residue after burning.

Subcutaneous injections of the aqueous solution of toxin into albino rats showed that the minimum lethal dose is 0·03 mg./100 g. body weight (average of twenty-four observations). A period of about 50–80 min. elapses between injection and death of animal. Boiling of the solution of toxin in water destroys its toxicity. One mg. of toxin when injected after boiling is harmless to rats. Incubation of the toxin solution at 37° C. for 24 hr. is sufficient to destroy its toxicity.

A solution containing 1 mg. toxin/ml. water gives positive reactions with Millon, xanthoproteic and biuret tests. Half-saturation with ammonium sulphate does not precipitate the toxin but it is precipitated by complete saturation.

An aqueous solution of the toxin, when treated with picric acid, gives an immediate yellow precipitate which dissolves on heating and reappears on cooling, suggesting that the toxin is a secondary proteose.

PHYSIOLOGICAL STUDY

(*a*) *Effect on the isolated uterus of the guinea pig.* The uterus of a virgin guinea-pig of about 250 g. body weight was excised and suspended in oxygenated Tyrode solution at a constant temperature of 37° C. The normal activity of the uterus was recorded. 0·2 ml. of a solution containing 1 mg. toxin/ml. Tyrode solution was added to the organ bath. An immediate contraction was obtained. The effect was persistent, but after washing the normal activity returned. The addition of 1 ml. of a solution containing 2 mg. of atropine sulphate in Tyrode solution abolished the excitatory effect of the toxin (see Fig. 1).

(*b*) *Effect on the isolated intestine of the rabbit.* The jejunum of a freshly killed rabbit of 1 kg. body weight was excised and suspended in oxygenated Tyrode solution kept at a constant temperature of 37° C. The normal activity was recorded. On addition of 0·2 mg. of toxin in Tyrode solution an immediate slight relaxation occurred followed by vigorous and persistent contraction. Several washings were necessary to abolish the effect. The addition of 1 ml. of atropine sulphate abolished the effect of the toxin, whether added before or after the toxin solution. The effect of bellafoline was similar to that of atropine.

(*c*) *Effect on the perfused frog's heart.* Isolated hearts of medium-sized frogs were perfused with Ringer–Locke solution. The normal activity of the heart was recorded.

The introduction of varying doses of the toxin into the perfusing fluid had the following effects:

(1) The heart rate was decreased.
(2) The height of contraction was increased.
(3) Partial block followed by complete block (Fig. 2) occurred. The heart stopped in diastole.
(4) In some experiments extrasystoles followed by compensatory pauses were observed.
(5) Washing abolished all the above effects and revived the heart (Fig. 2).

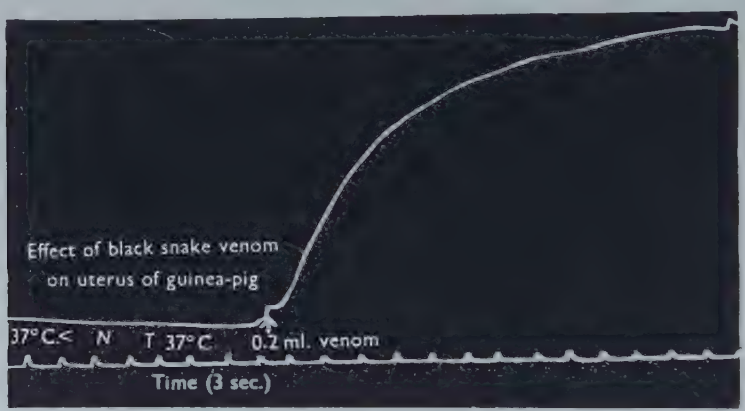

Fig. 1

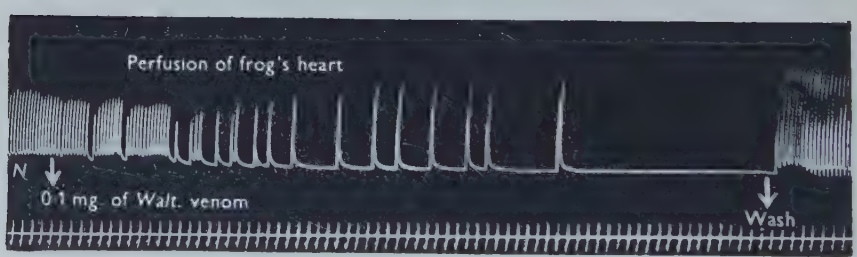

Fig. 2

The addition of 0·5 mg. of atropine sulphate to the perfusing fluid abolished these effects.

(*d*) *Effect on the perfused rabbit's heart.* Isolated hearts from freshly killed rabbits of an average weight of 1 kg. were perfused with oxygenated Ringer–Locke solution. After the normal activity had been recorded, 0·1 mg. of the toxin solution was introduced into the inflow rubber tube. After a latent period of 15–60 sec., changes similar to those observed in the case of the frog's heart occurred: slowing of the heart rate, increase in contractility, appearance of different grades of partial

heart block, complete heart block; and in some experiments extrasystoles followed by compensatory pauses were observed.

These effects were abolished by washing or by injection of 0·5 mg. of atropine sulphate (see Fig. 3).

(*e*) *Effect of salivary secretion.* One of the common symptoms observed after injection of toxin intravenously into rats and dogs was increased salivation. Accordingly, it was thought worth while to study the effect of injection of toxin on the rate of salivation in dogs anaesthetized by chloralose and with their salivary ducts dissected and cannulated. The number of drops of saliva collected per minute was taken as an index of the activity of the salivary gland.

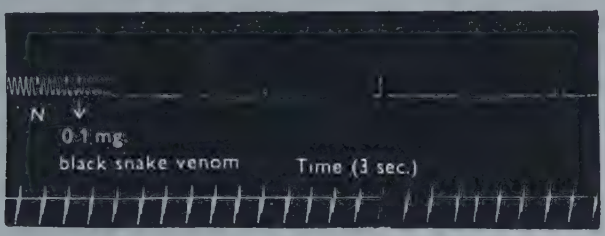

Fig. 3

In one experiment one drop was collected per minute. On stimulation of the chorda tympani by faradic current for a period of 30 sec. the rate of secretion rose to five drops per minute. Similar yields were obtainable on repeating stimulation after periods of rest. On the intravenous injection of 2 mg. of the toxin, the flow of saliva increased to seven drops per minute after a latent period of 8 min. The effect persisted for about 35 min. Stimulation of the chorda tympani after injection of toxin increased the salivary flow to twelve drops per minute. Intravenous injection of 1 mg. atropine sulphate abolished the effect of toxin, and no change in rate of salivary flow was observed. Ergotoxin injected intravenously had no effect on the rate of saliva flow after the injection of toxin.

These experiments were repeated with similar results.

DISCUSSION AND CONCLUSIONS

Most of the toxins from different venomous animals when examined chemically have been shown to be protein in nature (Ganguly & Malkana, 1936).

Biochemical study of the black snake toxin has shown that it is probably a proteose since when the aqueous solution of toxin is treated with picric acid it forms a precipitate which dissolves on heating but reappears on cooling.

The effects produced by crude venom are usually difficult to interpret. The possibility that the effect observed may be partially or entirely attributable to foreign contaminants cannot be excluded. Crude venom kept dry or in solution is known to decrease in potency with time (Guena & Calabrease, 1941). It

is therefore preferable to test the action of the venom when it is prepared in a pure crystalline form.

When the toxin is prepared in crystalline form its potency and activity are retained for a very long time. The minimum lethal dose can thus be reliably determined. In this way the toxicities of different venoms can be compared. The toxicity of the black snake venom was found to be ten times that of the Egyptian cobra venom (*Naja haja*).

It can be deduced from the experimental work on isolated organs of amphibia and mammals that this toxin has a parasympathetic effect. Its excitatory action on the isolated uterus of the guinea-pig and on the isolated intestine of the rabbit is abolished by addition of atropine. Again, the increase in the rate of saliva flow as a result of intravenous injection of toxin and the antagonistic effect of atropine are further indications of the parasympathetic effect. In this respect the black snake venom acts similarly to scorpion venom. Scorpion venom, however, has been shown to give an additional sympathetic effect (Mohammed, 1940).

Nevertheless, experiments showed that atropine sulphate when injected after injection of toxin did not save the life of the animal although it prolonged it. This suggests that the venom has another effect, probably a histamine-like action. Feldberg & Kellaway (1937) and Tretchewie & Kellaway (1940) have shown that histamine is liberated by perfused tissues when the venom of various snakes is added to the perfusing fluid. In the study of the Egyptian black snake it has been found that it causes histamine liberation from the skeletal muscles of the rat (Mohammed & Zaky, unpublished). In this respect the black snake venom behaves similarly to Egyptian bee venom (Karimi, 1955).

It is probable that the venom action is dependent on 'the route of administration' as has been pointed out by Shottler (1951).

The toxin interferes with the conducting system of the heart. It causes partial and complete block of the perfused amphibian and mammalian hearts. The toxin has also a direct effect on the myocardium since it causes augmentation of the heart beat. The venom may have another excitatory effect on the normally dormant ectopic centres in the walls of the ventricle since in some cases a series of extra systoles occurred.

It seems justifiable to believe that the venom has no damaging or irreversible effects on the different organs since simple washing of the poisoned organ is followed by complete recovery from all the above-mentioned effects.

SUMMARY

1. Two methods for preparation of the toxin in a purified crystalline form are described.

2. The toxin is probably a secondary proteose.

3. It has an excitatory parasympathetic effect on the uterus of the guinea-pig and on the intestine of the rabbit. The effect is abolished by atropine.

4. It causes excessive salivary secretion, again abolished by atropine.

5. It causes partial or complete block of perfused isolated amphibian and

mammalian hearts. It also causes extrasystoles. Both effects are abolished by atropine.

6. The minimum lethal dose for rats is 0·035–0·05 mg. toxin/100 g. body weight. Atropine did not save the life of the animal although it prolonged it.

7. The possibility of a histamine-like action of the venom is discussed.

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THE COVERING REACTION OF SEA-URCHINS

I. A PRELIMINARY ACCOUNT OF COVERING IN THE TROPICAL ECHINOID *LYTECHINUS VARIEGATUS* (LAMARCK), AND ITS RELATION TO LIGHT

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INTRODUCTION

It has long been known that several species of littoral sea-urchins clothe themselves with fragments from their surroundings. The reaction has been interpreted as a means of concealment by Schmidt (see Brehm, 1884), MacBride (1909), Boone (1925), as a defence against desiccation and temperature extremes (Orton, 1929), or as a reaction to strong light (von Uexküll, 1899; Dubois, 1914; Lindahl & Runnström, 1929; Mortensen, 1943*b*).

Lytechinus variegatus (Lamarck), a common littoral sea-urchin in the Caribbean, shows this habit strikingly (Field, 1892; Boone, 1925; Clark, 1933; Mortensen, 1943*a*), for in Jamaica large numbers are found almost completely concealed.

Such a clear manifestation of covering is obviously suitable for determining how it is performed and factors which influence it. A summary of the main findings has already been published (Millott, 1955).

THE MECHANISM OF COVERING

(*a*) *Taking up cover*

This is performed primarily by the tube feet. Most commonly those below the ambitus extend until they reach loose objects to which the terminal sucker adheres, and each tube foot then contracts so as to pull the material firmly against the tips of the primary spines which are then moved aborally (Fig. 1). The spines, acting on the crowbar principle, lever the material upwards aided by their roughened surfaces and the action of tube feet situated more aborally, so as to bring it in contact with the tips of neighbouring spines which lever it nearer the periproct. On reaching the aboral surface it is held there by the underlying tube feet, and by repetition of this process an extensive covering may be assembled.

The process resembles that described by Schmidt (see Brehm, 1884), as occurring in *Toxopneustes lividus* Agass.,* and contrary to the statements of Clark (1933), the pedicellariae take no part in seizing or holding the fragments.

* The account of the habit of this species, attributed to O. Schmidt, appears in the French edition of Brehm, *Les Merveilles de la Nature, Les Vers, Les Mollusques etc.* (ed. de Rochebrune, Paris, 1884), under the description of the species '*Toxopneustes lividus* Agass.'. In a reference to this account, Dubois (1914) designates the species '*Strongylocentrotus lividus* Brdt.'. These appear to be the species described in Mortensen's monograph (3, 3, pt. 2, p. 157), as *Paracentrotus lividus* (Lamarck).

Such behaviour may be elaborated when the urchin is subjected to directional or uneven illumination or when the pieces to be used for covering offer considerable resistance.

In the former case, the covering may be placed so as to shade the brightly illuminated areas of the surface, and when the urchin is turned, so that the covering no longer does this, either more covering is taken up or the existing covering is moved by co-ordinated action of the tube feet and spines until it regains its original position with reference to the light source. This behaviour recalls that described by Dubois (1914), as occurring in *Strongylocentrotus lividus* Brandt, and by von Uexküll (1899) in *Sphaerechinus*.

Fig. 1. Diagram showing a mode of action of the tube feet and spines, employed by *Lytechinus* in seizing and assembling as cover an empty gastropod shell (p. 508). The actions of two tube feet and one spine only are shown in successive stages I–III. Crossed arrows show the direction of movement of the shell; dotted arrows, the direction of the pull exerted by the tube feet; solid arrows, the direction of spine movement. I, extended tube feet are applied to the shell; II, the shell has been drawn to the test by the tube feet and bears against a primary spine; III, the spine is moved aborally and the shell levered upwards. Additional tube feet are extended as indicated by the dotted lines, the shell being held off the surface of the test by subjacent spines (not shown).

The orientation of covering appears very striking where an urchin is crossed by a narrow band of light (Fig. 2). Four stones taken up in three ambulacra were moved approximately along the routes shown so as to become placed along the band of light. Here the co-operation between the tube feet and spines in different ambulacra and interambulacra is noteworthy.

The number of feet used in assembling pieces of covering is roughly in direct proportion to the resistance offered by such fragments. Again, when a covering piece is heavy, or firmly wedged in the substratum, urchins may use greater force by moving their bodies against it, with the subambital spines held stiffly erect. Thus partly raised or loosened, the object is then lifted as previously described. Alternatively, whilst anchored by the oral tube feet, urchins may bring the tips of the subambital spines to bear against the surrounding stones, which are then loosened by a vigorous sideways movement of the spines.

Light floating or suspended objects such as dead leaves (or in the laboratory cover-glasses) may be seized by the aboral tube feet, pulled down on the neighbouring spines, held as covering here, or after being moved over the surface of the urchin, as described above.

The number of tube feet used varies considerably. One aboral tube foot is sufficient to seize a floating $\frac{7}{8}$ in. cover-glass and pull it below the meniscus, but usually when the floating object has been captured, the tube feet concerned are supplemented by others which adhere to it and assist in pulling it below the surface.

Since tube feet extend to capture the floating pieces, we may seek to find the type of stimulus involved.

That tactile stimuli are not significantly involved, and that a change in light intensity is important is shown by moving clear and enamelled cover-glasses over the

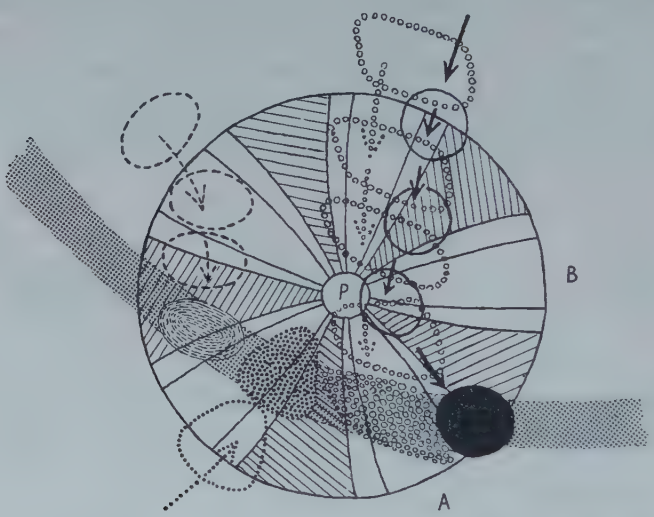

Fig. 2. Diagram showing the placing of cover over localized, brightly illuminated, areas of the surface (p. 509). Four stones, each shaded by a distinctive convention (lines, dots, circles, or solid black), are moved into a narrow band of sunlight (stippled) crossing the aboral surface of an urchin, approximately over the routes shown by successive outlines and arrows, drawn with the corresponding conventions. The interambulacra are distinguished by cross-line shading. The urchin endeavoured to take up stones from area *A*, but failed. No material suitable for cover was available in area *B*. *P*=periproct.

brightly illuminated aboral surface of naked urchins. Enamelled ones elicit extension of tube feet from the areas they shade, whereas the clear ones do not. Again, cover-slips enamelled with a simple pattern are usually seized by tube feet extended from the areas shaded by the pattern as shown in Fig. 3.

We may now ask whether the process previously described as occurring in continuous illumination may not have been due to shading, since urchins move about among stones, shells, etc., big enough to cast shadows on some part of their surface. That this is not so is shown by using lights mounted directly above urchins on a dull black background. Tube feet are extended and attached to shells, etc., despite the fact that the latter cast no shadow on the urchin. Again, where covering is moved over the surface, the shadows cast by it could be a factor bringing about extension of successive tube feet, but since clear cover-slips can be carried over the surface of the test and positioned in the same way as opaque objects, shadow cannot always be important.

The covering process is thus affected by light, but in one case by continuous bright light and in the other by a change in intensity. To what extent the two processes are distinct is not yet clear. Where urchins transferred from shade to bright

light take up covering they tend to do so initially when their surface is brightly and evenly illuminated, using the mechanism described on p. 508. Later, all tube feet exposed to light and not involved in holding the covering (see below) may be withdrawn, but by shading they can sometimes be induced to re-extend and take up covering.

Stimuli of other kinds may interfere with the extension of the tube feet. Thus touching a spine brings about instant withdrawal of any neighbouring tube feet extending into shadows, but if the tube feet are already attached by their suckers, they will not free themselves unless the spines are tapped vigorously and repeatedly. Again, the general behavioural state is important, for during active locomotion urchins usually cannot be induced to take up covering.

It now remains to discover how the covering is held in place.

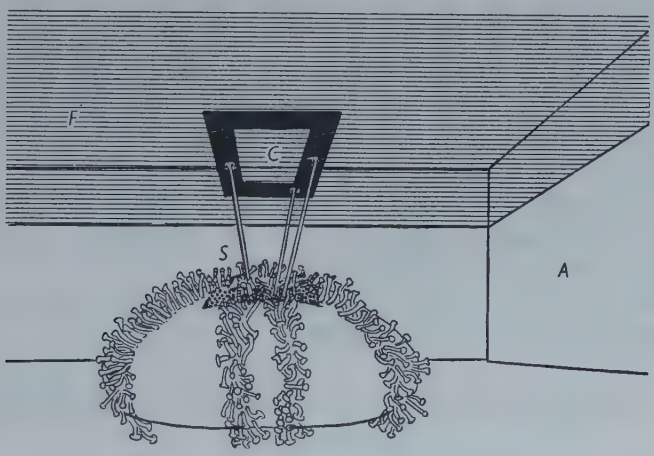

Fig. 3. The effect of a shadow cast on the surface of *Lytechinus* (drawn without spines and pedicellariae) which seizes a $\frac{7}{8}$ in. cover-glass (*C*), floating on the overlying surface film (*F*) of sea water, contained in a shallow dish with one side marked *A*. Note the three tube feet extended from the area covered by the shadow (*S*), cast by the opaque (enamelled) margin of the cover-slip, to which their suckers adhere (p. 510).

(*b*) *Holding cover*

Covering is maintained by prolonged contraction of tube feet which are assisted to varying degrees by the spines. This raises the question whether the same tube feet are involved the whole of the time. No satisfactory means was devised to answer this when opaque fragments were held, but it was possible to see what happened when the covering was transparent. When clear cover-glasses were placed over a part of the urchin's surface which had just been shaded, they were seized and retained as cover, even when the shaded areas were re-illuminated by two 50 c.p. lamps placed about 1 ft. away.

In one such experiment the cover-slip was initially held against the tips of the subjacent spines by the pull of nine tube feet originating from the aboral region of

one ambulacrum. Later they were supplemented (see below) and their suckers were disposed roughly in three groups A, B and C (Fig. 4). The cover-slip was held in this position for 17 hr., and though it was not possible to observe the tube feet all the time, continuous periods of observation of up to $1\frac{1}{2}$ hr. were possible.

The attached tube feet remained in the same three groups, but all the tube feet did not adhere permanently, some in each group were periodically withdrawn and replaced by others so that the total number of tube feet involved varied from 9 to 27. An idea of the total rate of change can be obtained from Table 1, and the rate of change in each group is shown in Table 2. Some tube feet remained attached to the cover-slip for $1\frac{1}{2}$ hr., while others beneath it took no part.

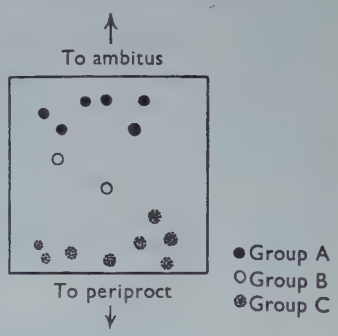

Fig. 4. The disposition of the suckers of tube feet attached to a $\frac{7}{8}$ in. clear cover-slip used as covering, at one stage of the experiment described above. Their arrangement in three groups (shaded distinctively) and the approximate orientation of the cover-slip are indicated.

Though the effective tube feet were subjected to abnormal conditions in being continuously and brightly illuminated, it is significant that opaque cover-slips were held by the same urchins alongside the clear ones. No difference in the way of holding the two types was observed, though we have no comparable details of the distribution and behaviour of the tube feet below the opaque cover-slips, because any attempt at lifting the cover to inspect such tube feet always resulted in some of them becoming detached.

Table 1. *Total number of tube feet used in holding one $\frac{7}{8}$ in. cover-slip between periproct and ambitus*

(Time to nearest half minute)

Time	No. of tube feet in use	Time	No. of tube feet in use
4.36 p.m.	9	4.51 p.m.	19
4.37	8	4.52	18
4.39	11	4.53	21
4.41·5	13	4.54	20
4.45·5	14	4.54·5	19
4.46	12	4.55	22
4·48	13	4.57	23
4.49	14	4.58	22

THE EFFECT OF LIGHT

Taxic responses and the responses of various organs to changes in intensity show that *Lytechinus* is affected by light.

The effect of light on certain tube feet has already been mentioned, but since these are primarily important in covering, their responses have received special attention and will now be described more fully.

(1) *Responses of the tube feet*

In most cases reactions were studied in the laboratory using young urchins and, as the light source, overhead tungsten lamps or 50 c.p. spot-lamps. The use of spot-lamps eliminates significant heating effects, for the urchins were invariably several inches below the surface of the water, and under such conditions two beams were found to raise the temperature of the water by but 1° C. in 45 min. Most experiments were of much shorter duration.

Table 2. *Rate of change of tube feet in the groups A, B and C (see Fig. 4)*

(Note: Owing to difficulties in observing and recording simultaneous changes in three groups, the following figures must be regarded as minimal. Time to nearest half minute.)

Time	Group	Change in no. of holding tube feet	Time	Group	Change in no. of holding tube feet
6.03	B	−1	6.33·5	B	+1
	C	+1	6.34	A	−1
6.05	A	−1	6.34·5	A	+1
6.08	A	+1	6.36·5	A	+1
6.09	A	−1	6·37	C	−1
	C	−1	6·42	A	−1
6.10·5	A	+2	6·44	A	−1
6.13·5	A	−1		A	+1
6.15	C	−1	6.45	C	−2
	B	+1	6.45·5	B	−1
	A	+1	6.48	A	+2
6.16	C	+1	6.49·5	B	−1
6.19·5	A	+1*	6·50	C	−1
6.20	A	−1*		B	−1
6.24	A	+1		B	+1
6.25	A	−2	6.51	A	−1
6.27·5	A	+1	6.52	A	−2
6.29·5	A	−1		B	+1
6.30·5	B	+1	6.52·5	C	+2
6.31	B	+1	6·53	A	+1
6.33	C	−1	6.54	A	−1
	A	−1	6.55	C	−1

* Same tube foot.

Tube feet respond to continuous light and to changes in intensity.

To the former they react variably, not only in different individuals but also in the same individual at different times. Thus tube feet may be unresponsive, withdrawn, extended without attachment or extended and attached to surrounding objects. Sometimes they withdraw immediately after a light beam is projected on to them, at other times they require more prolonged illumination from the same light source before withdrawing. Where tube feet appear unresponsive they may be induced to withdraw more quickly by increasing the light intensity; thus in one experiment 15 sec. illumination from one 50 c.p. lamp was required, but only 5 sec. from two such lamps placed at the same distance.

Responses both to increases and to decreases in intensity are more constant; the responses to shadows are the most constant and striking. In either case tube feet are quickly withdrawn and then, after a varying interval, slowly extended. If they

touch anything suitable they adhere and by contraction attempt to pull it on to the surface of the urchin, otherwise they wave or circle actively for 60–90 sec. before withdrawing again. Such reactions can be obtained repeatedly, and, further, follow too rapidly after the changes in intensity to be due to temperature changes.

Deep shadows are not essential. Thus when urchins were illuminated by two convergent beams focused on to the same area, cutting off one was sufficient to cause extension, despite the fact that the change in intensity at the surface of the urchin, as measured by a Weston 'Master II' exposure meter, was only from 20 to 13 units. Similarly, small colour filters (Ilford yellow-green no. 605, red no. 608 and violet no. 601) placed between the urchin and the light brought about extension.

During active locomotion, it is difficult to elicit any reaction in the tube feet by changing the light intensity; further, they appear relatively insensitive to contact stimuli and do not readily attach themselves, though sometimes the tube feet of the leading ambulacra only are so affected. When locomotion ceases the tube feet regain responsiveness in 13–17 min. Responses to increases in intensity sometimes appear some 10 min. before those to decreases.

When a narrow beam, projected on to one ambulacrum, is interrupted, the tube feet extend in neighbouring ambulacra, but in smaller numbers and less rapidly. Since the beam was narrow, the change in intensity in the neighbouring ambulacra was clearly less than in the illuminated one, and it was therefore suspected that there might be a relation between the speed of extension of the tube feet and the degree of change of intensity. The following experiments show that this is so.

Urchins were illuminated by three lamps arranged to shine directly on to one ambulacrum in which the tube feet were withdrawn. An opaque object was moved into the light path, and the time required for the tube feet to touch it was determined, first with one lamp, then with two and finally with all three. Since the background lighting was dim and the shading object opaque, throwing a shadow

Table 3

Expt. no.	Intensity of illumination expressed as an approximate scale reading of the photometer	Average time of contact of the first four tube feet in six successive trials (expressed in seconds)
1	300	50
	800	27
	1200	20
2	300	34
	800	17
	1400	11
3	200	25
	400	23
	700	17
4	200	23
	800	14
	1200	10

In experiments where temperatures were taken, the heating effects due to the spotlamps were found to be negligible, amounting to 1° C. in 90 min.

larger than the ambulacrum, it is safe to assume that the degree of intensity change increased with the number of lamps and roughly in the same measure as the intensity of the light they produced. The light intensity at the surface of the urchin was measured roughly by means of a Weston 'Master II' exposure meter. The shading object was held just beyond the tips of the longest spines projecting from either side of the ambulacrum, and so its distance from the test was approximately constant for each urchin. The average time required for the first four tube feet to touch it was determined in six successive trials. The results are shown in Table 3.

The rate of extension of the tube feet therefore increases with the degree of ntensity change.

There is a noteworthy difference in the reactions to shadows shown by the tube feet of *Lytechinus* and those of *Diadema*. In the latter, which does not cover, the reaction is simpler; the extended tube feet are usually flexed towards the substratum and then quickly recover (Millott, 1954).

(2) *Covering responses to diurnal changes in light intensity*

The extent to which urchins cover at various times throughout the day in their normal environment was determined. In view of the suggestions already made by previous workers (p. 508), it was essential to make observations in a locality where the urchins were never exposed to air nor subjected to considerable temperature change.

Such a locality exists in Jamaica, as a gully, through which passes a constant stream of sea water, the flow being such that the temperature in the mid-morning sunlight of December in the centre of the open stream was 27·5° C. and half a degree higher at the edges, while in the shade, that of both centre and edges was 27·5° C.

Some 3 hr. after sunrise, when about three-quarters of the gully was shaded from the sun, about 94% of the urchin population carried some covering, but there was a significant difference in the extent of the cover carried by urchins in the sunlit and shaded zones (Table 4). One hour later the shaded portion of the gully was

Table 4. *Percentage of population showing degree of covering indicated in the left-hand column*

(Note: in this and succeeding tables, urchins in which the aboral surface was largely concealed, are referred to as 'fully covered'.)

	Shaded zone	Sunlit zone
Without cover	13	nil
Partly covered	67	13
Fully covered	20	87

smaller and there were fewer naked urchins. In both shade and sunlight there was a greater proportion fully covered (Table 5).

With the fall in intensity at sundown, urchins shed covering (Table 6). Two hours after sundown the proportion of naked urchins living where the gully had previously

been exposed to the setting sun had increased over twofold, whereas in the part that had been shaded it remained about the same.

Table 5. *Percentage of population showing degree of covering indicated in the left-hand column*

	Shaded zone	Sunlit zone
Without cover	7	nil
Partly covered	63	5
Fully covered	30	95

Table 6. *Percentage of population showing degree of covering indicated in the left-hand column*

	Shaded zone	Zone illuminated by setting sun
Without cover	37	15
Partly covered	47	68
Fully covered	16	17

Thus covering is influenced by light intensity, though a surprisingly large proportion (about 60%) of the urchins retained some covering in darkness.

This can also be shown in the laboratory where many urchins continue to cover in captivity, but their covering is not so extensive nor is it usually held for so long as in their natural surroundings, even when fragments normally employed as covering are used. Thus many urchins living in glass aquaria lighted by an east window begin to pick up cover about 1 hr. after sunrise, slowly shedding it in the fading light of the late afternoon.

The tendency to cover is most marked in the 3 hr. after sunrise, which suggests that the preceding sojourn in darkness may have increased responsiveness to light. This is confirmed by keeping urchins which have become unresponsive in darkened dishes for 4–16 hr., after which they often cover again.

(3) *Covering responses to artificial light*

Covering can sometimes be induced by one or more 50–100 W. tungsten filament lamps, either alone at night or added to sunlight at various times of the day. Heating effects can be discounted, for the aquaria were large and covering was completed about 5 min. after the light was switched on. Conversely, covered urchins transferred to darkness may shed their covering.

Differences between individuals are very great; some urchins could not be induced to cover in strong light, but moved into shade, while a few retained their covering in darkness for as long as 19 hr.

In general, however, the covering behaviour of urchins under experimental conditions agrees with that observed in their normal surroundings and shows the

influence of light, though the range of variation would suggest that some urchins may become less sensitive to it in captivity. Again, the possible existence of an inherent physiological rhythm should not be overlooked, but the effect of light is sufficiently clear to show that if such a rhythm exists it is by no means the only factor involved.

This variation in behaviour toward light recalls that encountered in sea-urchins such as *Arbacia* (Holmes, 1912) and *Diadema* (Millott, 1954). In the latter, as in *Lytechinus*, sensitivity to light varies with body colour, though *Diadema* shows physiological colour change (Millott, 1952) which I have not seen in *Lytechinus*.

(4) *The influence of body colour*

The predominant colours of *L. variegatus* are green and white, varying in relative proportion as well as in the depth of the green colour, so that pale, dark and intermediate individuals can be distinguished.

Animals of all shades cover in their natural surroundings, and I found no difference in the extent of their covering. However, pale urchins stripped in the mid-morning sunlight tend to cover again more readily and quickly than dark ones similarly treated, so that in a sample containing both kinds the pale individuals picked up covering within 4 min., whereas the dark did not begin until some 9 min. later. Again, when the illumination of an indoor aquarium was increased by two overhead lamps (100 and 75 W.) all the pale urchins, together with a few dark ones, began to cover, while a greater number of dark ones remained uncovered. Usually, in well-lighted aquaria where urchins remain uncovered, there is a preponderance of dark individuals among them, and unless they are subjected to direct sunlight dark individuals carry less cover than pale ones.

The reason for this is obscure, but from purely physical considerations the green pigment would appear not to be involved directly in any photoreceptive process which leads to covering, otherwise the opposite would be expected. It could serve as a screening pigment, but if this were so, since light influences dark forms as well as pale, it would appear that the pigment is either insufficient or absent from some, at least, of the light-sensitive areas. The existence of such screening pigment has already been demonstrated in the skin of *Diadema* by Millott (1954). Much earlier von Uexküll (1897, 1900) suggested that the pigment in the skin ('Pigmenthülle') of echinoids such as *Centrostephanus* serves as a light screen, and he noted that species lacking such protection cover themselves with debris. In *Lytechinus*, however, the differences between dark and pale forms are not necessarily due solely to differences in the amount of green pigment. There is at least one reddish pigment and there may be others masked by the green.

(5) *The effect of photosensitizing dyes*

The importance of light in relation to covering is also shown by injecting photosensitizing dyes dissolved in sea water or coelomic fluid into the perivisceral coelom. The dyes used, rose bengal (tetrachloro (P) tetraiodo (R)-fluorescein), eosin Y (tetrabromo-fluorescein) and neutral red are known to photosensitize (Metzner,

1927; Welsh, 1934), and are marketed respectively by Messrs B.D.H., the National Aniline Division of the Allied Chemical and Dyeing Corp., N.Y. and G.T. Gurr (their 'vital' grade). Controls were set up in which similar amounts of sea water or coelomic fluid alone were injected. No attempt was made to standardize the amount of dye used. The injected urchins and the controls were kept in glass aquaria and provided with ample material with which to cover.

The results were striking. Urchins injected with dye began to cover about 2 hr. after injection, and when stripped of covering re-covered themselves immediately even in fading daylight, while the controls remained naked. About 1 hr. after sunrise the injected urchins showed abnormally great activity, their tube feet extending in all directions, those below the ambitus rapidly picking up covering while those of the controls were still inactive. Activity continued until the urchins were completely buried while the controls remained uncovered. Covering was extensive, whether the injected urchins were pale or dark, exposed or in the shade, and it was retained until after sundown; but they would not cover in darkness. The effect lasted up to 7 days depending on the amount of dye used; urchins could be resensitized by one or two subsequent injections, but too much dye proved toxic. The effect was sometimes observed in urchins which had been kept in aquaria for a number of days and which had ceased to cover.

Neutral red brought about an effect similar to that of rose bengal and eosin Y; safranin (water soluble, G.T. Gurr) and methylene blue (vital, G.T. Gurr), did not enhance the covering response, the former appearing toxic to the urchins.

The effective dyes when dissolved in sea water absorb extensively between 410 and 565 mμ (Fig. 5). If this is roughly the same when the dyes become associated with the living matter, then in view of the photosensitizing effect of the dyes it follows that the naturally occurring pigments cannot effectively screen all the living matter from these wave lengths.

Among the dyes used that were likely to affect oxidation-reduction potential, neutral red was effective but not methylene blue. This may indicate that their possible effect on oxidation-reduction balance has little direct significance here, though they are poised at very different levels.

It is noteworthy that photosensitization could influence the relatively complex co-ordinated activity of covering, and thus the foregoing experiments strengthen previous indications that covering is influenced by light. They also show that the energy quanta normally available in the environment are adequate to excite the response and that pathways exist for the transfer of energy from mechanisms affected by such light to a co-ordinated system of effectors. Such mechanisms may or may not operate in the normal animal, for the use of such dyes may create artificial light-absorbing mechanisms. It is simplest to assume that the dye augments normal photoreceptive mechanisms, but it might have created others, for example, by making the effectors or intermediate elements such as nerves light-sensitive, as has already been shown possible by Lillie (1924), Lippay (1929) and Auger & Fessard (1933). The relation of such photosensitive processes to normal biological mechanisms is thus obscure (see also Blum, 1941; Blum & Kauzmann, 1954).

Nevertheless, in *Lytechinus*, these dyes affect activities that are normally influenced by light such as withdrawal of tube feet and movements of pedicellariae, as well as the covering reaction, more obviously and regularly than the other activities that were observed. Again, many of the injected urchins survived in captivity as long as their controls.

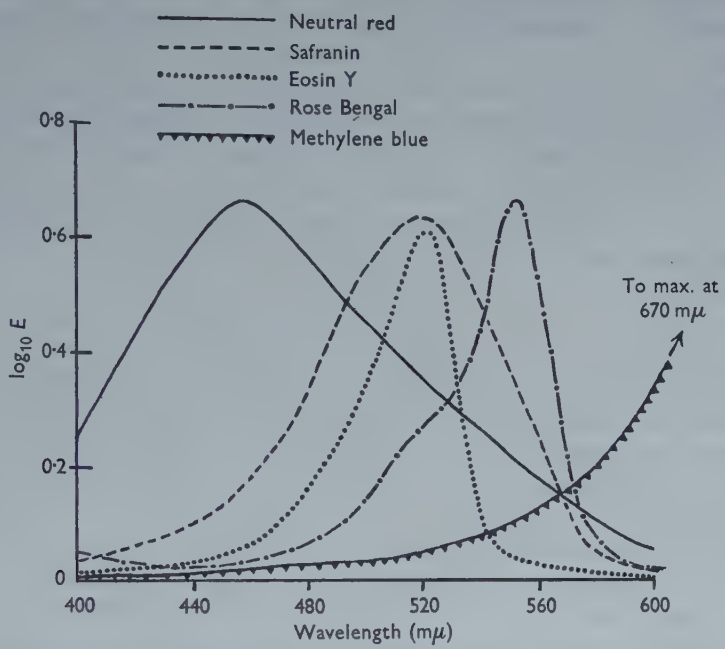

Fig. 5. Comparative light absorption, in the wave length range 400–600 mμ, of the dyes used in the photosensitization experiments reported on pp. 517 and 518. For the purpose of comparison the dyes were dissolved in sea water and the concentration adjusted so that the maximum absorption in the visible range of the spectrum was approximately the same in each case.

The responses of the tube feet to light and more especially the responses to shadows are, in general, what would be expected in view of the tendency to cover during continuous bright light and after changes in intensity (p. 510). Their variability and the influence of other activities indicates that covering movements are integrated into general behaviour and are not merely the result of simple responses of the tube feet to light. A few simple experiments were performed to discover whether the central nervous system is involved.

SIGNIFICANCE OF THE NERVE RINGS

No detailed descriptions of the nervous system of *Lytechinus* are known, but from our knowledge of the disposition and working of the system in other echinoids (Romanes & Ewart, 1881; Romanes, 1885; Fredericq, 1876; Delage & Hérouard, 1903), we may suspect that a circum-oral and perhaps an aboral nerve ring are involved in the covering reaction.

In the first type of experiment the lantern with the peristome and surrounding test was excised, and the urchin was replaced in a sunlit area of its normal environment. It slowly covered itself with stones. An oral nerve ring is thus not essential. In the second, the periproct and surrounding regions of the test were removed before replacing in sunlight. Covering was taken up as usual, the pieces being moved aborally as far as the cut edge of the test and held there. An aboral nerve ring is thus not essential.

These experiments are insufficient to eliminate completely central nervous coordination of covering movements, for the radial nerve cords were still intact and, further, the capacity of mutilated urchins to orientate covering with respect to the light source, or to move pieces of covering over the various routes seen in the intact animal (p. 509), was not demonstrated. Further observations and experimental analysis are clearly required.

DISCUSSION

It is clear from the foregoing account that the covering habit of *Lytechinus* is related to light. The same has been maintained concerning covering in other urchins by von Uexküll (1899), Dubois (1914), Mortensen (1943*a*, *b*) and Cuénot (1948). In a brief reference to the habit in *Lytechinus variegatus*, Clark (1933) remains noncommittal, but Boone (1925) rejects the significance of strong light, stating that individuals kept for several weeks in relatively dark indoor aquaria covered as thoroughly as those on open reefs. I cannot confirm Boone's observations (p. 516), nor did I succeed in keeping *Lytechinus* healthy in indoor aquaria for more than 2 weeks, but the effect of light on covering in the natural surroundings (p. 515) is clear enough.

In *Lytechinus* both continuous bright light and changes in intensity can induce the covering response. This is noteworthy, since photic stimuli of both types are known to elicit responses of a different kind in other echinoids (Millott, 1954). Whether other environmental changes can induce covering in this species is unknown.

Such characteristic and well-defined behaviour is likely to be in some measure adaptive and to have a definite selective value. It should therefore be related to some particular environmental requirement, and since the habit is common in littoral urchins we should seek factors that operate particularly in shallow water.

Temperature extremes and desiccation can be eliminated, since *Lytechinus* is tropical or subtropical and has always been immersed in the situations where I have found it. The mechanical effect of wave action can be discounted, for not only is the habit commonly displayed where such action does not exist, but it is doubtful whether covering would afford real protection for the surface of the urchin; it might even be a disadvantage, for covered urchins when displaced (as they could be by waves) are top heavy so that righting is difficult.

Light is a significant factor in shallow water, especially where it is intense as in the tropics.

Indirectly, it may make an animal conspicuous. Covering might prevent this and so has been interpreted as a means of advantageous concealment in *Lytechinus* (Boone, 1925). There is not sufficient evidence for this, for I have yet to find animals which prey upon this urchin; and though they may exist, predators with sufficient appreciation of form and pattern, as well as discrimination of intensity and colour, must be known before such views can be accepted. The same applies to concealment from potential victims, especially as it is by no means clear what the urchin feeds on; Boone describes a diet chiefly of 'small molluscs, crustaceans and worms', while Mortensen (1943*a*) finds the food to consist of 'bottom material, with shells and bits of plants'.

In a brief reference to covering in this species, Mortensen (1913*a*), expresses similar scepticism.

Light acting directly appears sometimes to be a nociceptive stimulus to *Lytechinus* (p. 513), suggesting that the animal has insufficient protection. An opaque covering acting as a light-shield would therefore confer an advantage, increasing the distribution range of the species by enabling individuals to seek food with impunity in shallow sunlit waters. The idea gains strength from the fact that there are vast numbers of this species in Kingston harbour, and like Field (1892) I have found that dredges rapidly become filled with them. It therefore seems reasonable to suppose that intraspecific competition, at least, is keen and that population pressure in slightly deeper waters is considerable.

The idea of covering acting as a light-screen in other echinoids has been advanced by Dubois (1914), Lindahl & Runnström (1929), and Mortensen (1943*a*, p. 389, 1943*b*, pp. 135 and 210).

In the instance of *Centrostephanus* von Uexküll (1897) goes further in suggesting that screening pigment might be a means of preserving photolabile visual pigment in the skin.

The same might apply to covering, but such an idea lacks sufficient evidence. Nevertheless, there are indications of photolabile pigments in echinoderms (von Uexküll, 1897; Crozier, 1915, 1920; Millott & Vevers, 1955), though carotenoids were found only in traces in the skin of *Lytechinus pictus* (Fox, 1953).

SUMMARY

1. *Lytechinus variegatus* (Lamarck) covers the parts of its skin that are exposed to light with fragments taken from its surroundings.

2. The covering is taken up by the tube feet, assisted by the spines, and held in place by the tube feet acting in relays. It may be orientated with respect to the light source. There are indications of adaptability of behaviour where the covering pieces offer resistance to being lifted.

3. Covering is related to light and to diurnal light changes, being assumed in strong light and rejected, after a varying interval of time, in darkness. Both continuous bright light and decreases in light intensity evoke covering. The tube feet react to the same stimuli and the speed of their extension is roughly proportional to the change of intensity.

4. The tendency to cover is increased after a sojourn in darkness and is greater in pale individuals than in dark ones.

5. Urchins can be photosensitized by injection of dyes so that they cover in dim light.

6. The prehension and holding of covering does not involve the oral and aboral nerve rings.

7. The relation of covering to light and environment favours the idea that it acts as a screen against strong light.

My thanks are due to Dr C. F. A. Pantin, F.R.S., for valuable criticism and advice, to my daughter Susan, who helped with the experiments recorded on p. 514, and to the Rockefeller Foundation, who generously provided the spectrophotometer used to determine the light absorption of the photosensitizing dyes.

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THE MECHANICAL PROPERTIES OF THE CELL SURFACE

IV. THE EFFECT OF CHEMICAL AGENTS AND OF CHANGES IN pH ON THE UNFERTILIZED SEA-URCHIN EGG

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INTRODUCTION

The earlier papers of this series (Mitchison & Swann, 1954*a*, *b*, 1955) described measurements of the rigidity of the cell surface of sea-urchin eggs which were carried out with an instrument called a 'cell elastimeter'. The present paper describes experiments using the same technique on unfertilized sea-urchin eggs which had been subjected to the action of various chemical agents and to changes in pH. The aim of this work was to discover something of the structural components of the cell membrane or cortex by testing the action of chemicals which might be expected to affect these components. If, for example, there were sulphydryl groups and disulphide links in the structural protein of the membrane, a reducing agent might be expected to decrease the rigidity and an oxidizing agent to increase it. There is also the possibility that the maintenance of the membrane structure might require energy from respiration, and therefore that respiratory inhibitors might alter the rigidity. Considerations such as these largely dictated the choice of the chemicals that were used, though a few others were tested for different reasons.

It should be said at the outset that the results of this work are difficult to interpret in view of our present lack of knowledge about the components of the cell surface. Many of the chemicals did not affect the membrane rigidity, and of those that did so only some changed it in a way that would be expected from simple theory.

MEASUREMENTS ON THE EFFECT OF CHEMICAL AGENTS

The materials used for these experiments were the unfertilized eggs of two species of Mediterranean sea-urchin, *Sphaerechinus granularis* and *Paracentrotus lividus*. No difference was found between the eggs of these two species in their behaviour towards any of the chemicals tested. The jelly was removed by pre-treatment with acidified sea water.

In the initial tests one sample of eggs was placed in the solution of the chemical agent and another sample from the same female was left in sea water as a control. After 3 hr. the rigidity of five eggs from each sample was measured with the cell elastimeter, using the technique described by Mitchison & Swann (1954*a*). If the difference between the average rigidities of the two samples was less than 20% it

Table 1. *Effect of chemical agents on the rigidity of the cortex of unfertilized eggs*

M.E. = Elevation of fertilization membrane.
Cyt. = Cytolysis.

Agent	Effect on rigidity	Max. conc. tested (molarity, unless otherwise stated)	Comments
1. Oxidizing agents:			
Hydrogen peroxide	No	10^{-2}	M.E. with 10^{-1}M
Sodium metaperiodate	No	10^{-4}	M.E. with 10^{-3}M Ca- and Mg-free sea water
Sodium iodosobenzoate	No	Sat. soln.	
Oxidized glutathione	No	10^{-2}	
Cystine	No	Sat. soln.	
Iodine in potassium iodide	Yes	10^{-4}	
2. Reducing agents:			
Sodium sulphide	Yes	10^{-2}	
Dithiopropanol (B.A.L.)	Yes	10^{-1}	
Sodium thioglycollate	Yes	10^{-2}	
Reduced glutathione	No	10^{-2}	
Cysteine (+sodium cyanide)	No	10^{-2}	Cyt. with 10^{-1}M
3. Mercaptide-forming agents:			
Phenyl mercuric acetate	No	Sat. soln.	
p-Chloromercuric benzoate	No	Sat. soln.	
Sodium arsenite	No	10^{-1}	
Sodium cacodylate	No	10^{-1}	
4. Alkylating agents:			
Chloracetophenone	Yes	10^{-3}	
n-Ethyl maleimide	No	10^{-4}	M.E. with 10^{-3}M
5. Respiratory inhibitors:			
Sodium cyanide	No	10^{-1}	
Sodium azide	No	10^{-1}	
Sodium fluoride	No	10^{-2}	Cyt. with 10^{-1}M Ca- and Mg-free sea water
Sodium malonate	No	10^{-1}	
Sodium iodoacetate	No	10^{-2}	Cyt. with 10^{-1}M
Iodacetamide	No	10^{-2}	
2, 4-Dinitrophenol	No	5×10^{-3}M	
6. Detergents:			
Cetyltrimethyl ammonium bromide	Yes	0·001 %	Cyt. with 0·01 %
Sodium dodecyl sulphate	Yes	0·001 %	Cyt. with 0·1 % M.E. with 0·01 %
$H(CH_2)_{10}(CH_2.CH_2.O)_8H$	Yes	0·01 %	Cyt. with 0·1 %
7. Salts:			
Sodium chloride	No	0·55	
Potassium chloride	No	0·55	
Magnesium chloride	No	0·37	
Calcium chloride	No	0·37	
Zinc chloride	Yes	—	
Cupric chloride	No	—	
Lead chloride	No	—	
Ferric chloride	No	—	
8. Miscellaneous:			
Formalin	Yes	10^{-1}	
Trypsin	Yes	0·1 %	
Ether	No	10 % sat. soln.	Cyt. with sat. soln.
Chloroform	No	10 % sat. soln.	Cyt. with sat. soln.
Glycine	No	10^{-1}	
Versene	No	10^{-3}	
Heparin	No	0·1 %	
Hyaluronidase	No	10 units/ml.	

was assumed that the test was negative and that the chemical had no effect on the membrane rigidity.

Table 1 gives a list of the chemical agents used and the maximum concentrations at which they were tested, and it also shows whether or not they affected the rigidity. The chemicals are divided into eight groups of which the first four are those that affect sulphydryl groups or disulphide bonds. The separation into the groups is somewhat arbitrary, since some of the respiratory inhibitors affect sulphydryl groups (e.g. iodoacetate) and vice versa. All the chemicals were made up in sea water except for sodium fluoride and metaperiodate. These precipitated in ordinary sea water and had to be made up in an artificial sea water without calcium and magnesium and containing only sodium and potassium chloride. The controls for these chemicals were also placed in this artificial sea water. The pH of all the solutions was measured with a glass electrode and, when necessary, adjusted to pH 7·5–8·0 with NaOH or HCl. The maximum concentration which was tested is given in most cases as molarity, but one-tenth saturated solutions were used with ether and chloroform, and fully saturated solutions with chemicals of low solubility (iodoso-benzoate, cystine, phenyl mercuric acetate and chloromercuric benzoate). In the case of zinc, copper, lead and iron, the chloride was made up at 10^{-2}M, but most of the metal precipitated as hydroxide when the pH was raised to 8·0 and the final concentration, after filtering off the precipitate, was probably very low. The chlorides of sodium, potassium, magnesium and calcium were made up in solutions which were approximately isosmotic with sea water. Table 1 also shows that some of the chemicals produced cytolysis or caused the elevation of fertilization membranes.

Further measurements were made with the eleven chemicals that produced definite changes in rigidity. An experiment was carried out with each of these chemicals to find the change in rigidity with time at different concentrations. Eggs were placed in a series of solutions of ten-fold dilution (e.g. 10^{-2}, 10^{-3}, 10^{-4}M) and in sea water as a control, and then measured (average of five eggs) every 30–60 min. for a period of 3–4 hr. The results for sodium thioglycollate are shown as an example in Fig. 1. There is no significant effect at a concentration of 10^{-3}M (or at lower concentrations which are not shown), but at 10^{-2}M there is a fairly steady rise in the rigidity. As in the earlier papers of this series, the rigidity is given as 'corrected stiffness' (dynes/cm.^{2}/μ deformation for the standard condition of 100μ diam. egg and 50μ diam. pipette). Some of the eggs were removed from the 10^{-2}M-thioglycollate after 130 min. and washed three times with sea water. They were then left in sea water and measured later. The rigidity dropped to a value nearly as low as the controls, thus showing that most of the effect of thioglycollate could be reversed by washing.

Of the remaining ten chemicals, seven caused an increase in rigidity and three caused a decrease. Excluding for the moment the case of trypsin, the general shape of the curves was similar to those with thioglycollate. They have not therefore been reproduced in detail, but the most important information from them is given in the first two columns of Table 2. The 'minimum concentration' is the most dilute of the ten-fold dilutions which showed an effect on the rigidity. This is not strictly

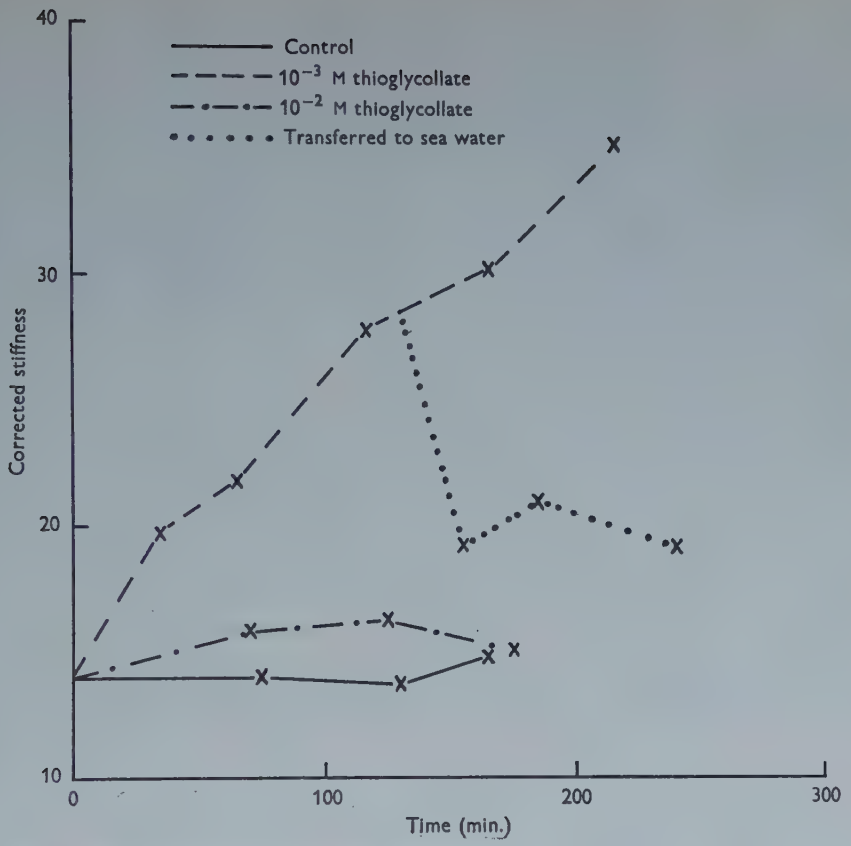

Fig. 1. Changes of membrane rigidity with sodium thioglycollate. Corrected stiffness is in dynes/cm.^{2}/μ deformation for 100 μ diam. egg and 50 μ diam. pipette.

Table 2. *Chemical agents with positive effect on cortical rigidity*

Agent	Min. conc. for effect	Change in rigidity after 3 hr. in min. conc.	Reversibility	Increased cytoplasmic rigidity
Oxidizing agent:				
Iodine in potassium iodide	10^{-4}M	×5·3	No	Yes
Reducing agent:				
Sodium sulphide	10^{-2}M	×2·2	No	Yes
Dithiopropanol (B.A.L.)	10^{-3}M	×6·7	Yes	No
Sodium thioglycollate	10^{-2}M	×2·1	Yes	No
Alkylating agent:				
Chloracetophenone	10^{-3}M	×3·8	No	No
Detergents:				
Cetyltrimethyl ammonium bromide	0·0001 %	×0·59	Yes	No
Sodium dodecyl sulphate	0·001 %	×0·44	Yes	No
$H(CH_2)_{10}(CH_2.CH_2.O)_8H$	0·01 %	×1·6	Yes	No
Salt:				
Zinc chloride	—	×1·6	Yes	No
Miscellaneous:				
Formalin	10^{-2}M	×8·3	No	Yes
Trypsin	0·001 %	×0·23	No	No

speaking the true minimum concentration, since there is a factor of ten between this concentration and the next more dilute concentration which showed no effect. At this minimum concentration the rigidity increased (or decreased) fairly steadily, though there was usually a more rapid increase in the first hour than there was subsequently. The second column of Table 2 shows the change in corrected stiffness after 3 hr. at this concentration as compared with the controls. With concentrations lower than the minimum, and with the controls, the rigidity remained nearly constant throughout the experiment. With concentrations higher than the minimum either the eggs showed larger changes in rigidity or measurements were impossible because of cytolysis or membrane formation.

The third column of Table 2 shows whether or not the rigidity change could be reversed by washing. A sample of eggs was removed from the minimum concentration of the chemical after a measurement at $2\frac{1}{2}$ hr., washed three times, left in sea water for a further hour and then measured again. If the rigidity had decreased (or increased) to the value for the controls the effect was regarded as reversible, while if it remained at its original value the effect was regarded as irreversible.

If the measured rigidity decreases this is almost certainly due to a decrease in the elastic modulus of the surface, since the interior cytoplasm of an unfertilized egg is so fluid that a decrease in its 'elasticity' would be most unlikely to affect the elastimeter readings. If, however, the measured rigidity increases, it might be caused by an increase in the elastic modulus either of the membrane or of the cytoplasm. It was therefore necessary to test whether those chemicals which affected the measured rigidity also caused an increase in cytoplasmic rigidity. The eggs were left in the minimum concentrations of the chemicals for 2 hr. and then centrifuged in a sugar gradient (1·1M-sucrose) at 7000 g. for 10 min. They were examined under a microscope and the degree of stratification of the granules was compared with that in control untreated eggs. With iodine, sodium sulphide and formalin, there was no stratification, thus showing that the cytoplasmic rigidity had increased. With the other chemicals there was no difference between the treated eggs and the controls. These results are presented in the last column of Table 2.

The effect of trypsin is shown in Fig. 2. These results differed in two respects from those with any of the other chemicals. First, an increase in the concentration made only a small difference to the decreased rigidity which was produced. Secondly, there was little change in the rigidity with time after the initial decrease. These effects will be discussed below.

MEASUREMENTS ON THE EFFECT OF CHANGE IN pH

The results of three experiments on effect of changes in the pH are shown in Fig. 3. Measurements of rigidity were made (average of five eggs) on samples of eggs from one female which had been left for 100 min. in sea water whose pH had been altered with NaOH or HCl and measured with a glass electrode. The first two experiments were made without buffers and there were slight rises in the pH (maximum of 0·2) during the course of the experiment. The pH given in Fig. 3 is the final pH of the solutions. The untreated sea water had a pH 8·0–8·1. The third

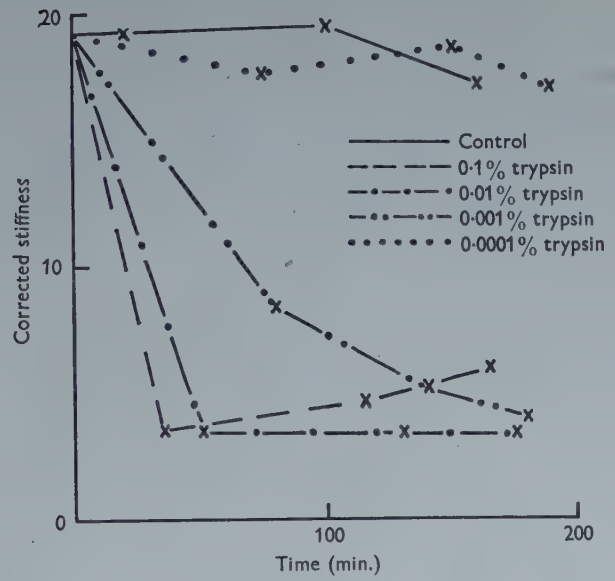

Fig. 2. Changes of membrane rigidity with trypsin. Corrected stiffness is in dynes/cm.$^2/\mu$ deformation for 100 μ diam. egg and 50 μ diam. pipette.

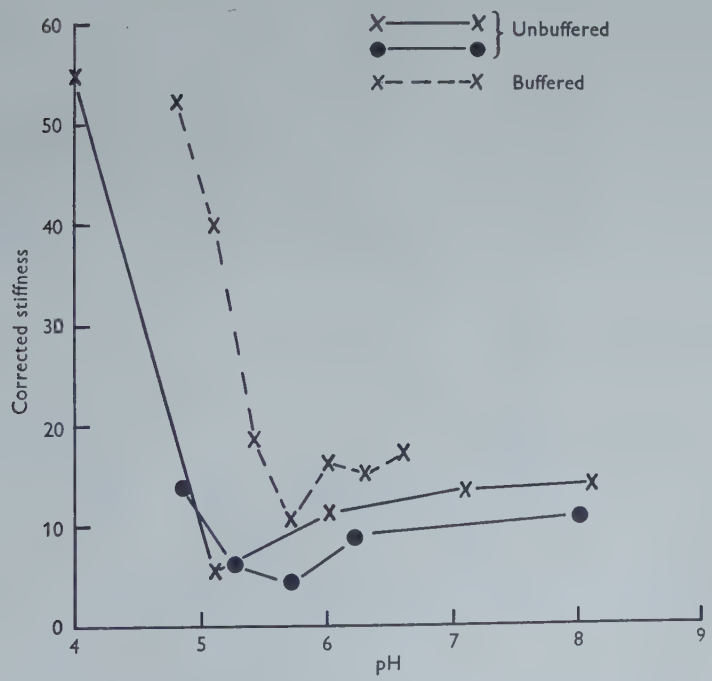

Fig. 3. Changes of membrane rigidity with pH. Corrected stiffness is in dynes/cm.$^2/\mu$ deformation for 100 μ diam. egg and 50 μ diam. pipette.

experiment was carried out with an artificial Ca- and Mg-free sea water and citrate-phosphate buffers. These solutions had constant pH values but were not very satisfactory media for the eggs, since there was an appreciable amount of cytolysis.

These results show a minimum rigidity about pH 5·7, and a sharp rise in rigidity in acid solutions with pH values of about 5·0 or less. The changes with time, which are not shown in Fig. 3, resembled those with trypsin in having a large initial change and thereafter little alteration. The high rigidity in acid solutions could not be reversed by washing.

DISCUSSION

Of the chemicals which affect the rigidity, formalin, iodine and sulphide cause an irreversible increase in the general rigidity of the cytoplasm and probably also in that of the cell membrane. This action is not surprising in the case of formalin, since it is well known to increase the rigidity of protein gels. It is worth pointing out that this method of determining rigidity might prove useful in a general study of fixation, since it provides quantitative measurements on a cellular level of one of the most important actions of a fixative.

The other chemicals which affect the rigidity appear to act mainly if not exclusively on the cell membrane or cortex. Apart from zinc chloride, they can be divided into three groups; SH reagents, detergents and trypsin. They will be discussed in this order.

Of the SH reagents that were tested, two of the reducing agents (dithiopropanol and thioglycollate) and one of the alkylating agents (chloracetophenone) affect the membrane rigidity. Dithiopropanol and thioglycollate are stronger reducing agents than cysteine or reduced glutathione and might be expected to have a more marked effect. It is, however, surprising that they increase the membrane rigidity. The simplest expectation would be that they would decrease the rigidity by breaking disulphide cross-links. The absence of any effect with the oxidizing agents suggests that there are few if any SH groups which can be converted into disulphide links, but if this is so it is difficult to explain the action of chloracetophenone which should react with free SH groups. It seems better not to draw any conclusions from these results in view of the fact that only a few of the SH reagents affect the rigidity and those that do affect it behave in an unexpected way.

Three detergents were tested; a cationic one (cetyltrimethyl ammonium bromide), an anionic one (sodium dodecyl sulphate) and a nonionic polyethanoxide ($H(CH_2)_{10}(CH_2.CH_2.O)_8H$). They all affect the membrane rigidity reversibly, though the cationic detergent is more effective than the anionic, and the anionic is more effective than the nonionic. This is the same order as that shown in their bactericidal properties (Putnam, 1948). The polyethanoxide increases the rigidity whereas the other two detergents decrease it. This may perhaps be connected with the fact that both cationic and anionic detergents combine with globular protein and cause a partial unfolding of the molecule (Few, Ottewill & Parreira, 1955). It is worth pointing out that all the detergents affect the membrane at concentrations between 1/10 and 1/100 of those which cause cytolysis.

The effect of trypsin differs from that of all the other reagents in that it appears to cause an 'all or none' reaction. As long as the initial concentration of trypsin is above about 0·0001%, the membrane rigidity drops irreversibly to a low value which is more or less independent both of time and of concentration of trypsin. This result would agree with the suggestion put forward by Runnström, Monné & Broman (1943) that trypsin digests away a vitelline membrane round the surface of unfertilized eggs. On the other hand, there is as yet no sign of a vitelline membrane under the light microscope or the electron microscope (Mitchison, 1956), so it may be that trypsin acts by disorganizing an outer layer of the cortex rather than by digesting away a separate membrane.

The experiments with varying pH show that there is a minimum rigidity at a pH of about 5·7. This may indicate that the isoelectric point of the membrane protein is at this value but, as with SH reagents, this is not the result that would be expected. At the isoelectric point there will be a maximum number of charged groups on the proteins, and these should produce an increased rigidity due to electrostatic forces between the molecules. On the other hand, Gerngross (1926) found that the rigidity of a gelatine gel was independent of pH over a range of about three pH units near the isoelectric point. This suggests either that the total number of charged groups is not greatly altered over this range, or that electrostatic forces are not of primary importance in determining the rigidity of a gel. In any case, however, there is always the difficulty with living cells that changes in the external pH may not produce equivalent changes in the pH of the membrane protein within the cell's permeability barrier.

Most of the reagents which were tested had no effect on the rigidity, but it is worth emphasizing two points about these negative results. First, the fact that none of the respiratory inhibitors affect the membrane rigidity indicates that the maintenance of the membrane structure does not depend on a supply of energy. Secondly, the fact that the rigidity remains unchanged both in solutions containing large amounts of calcium (e.g. 0·37M-$CaCl_2$) and in solutions free of calcium (e.g. 0·55M-NaCl) indicates that the main structural protein of the membrane is unaffected by changes in the external concentration of calcium ions. There is, however, a warning which should be given about all the negative results. The absence of an effect may simply be due to the fact that the reagent does not penetrate the egg. It is likely that the respiratory inhibitors penetrate since they stop division in a fertilized egg, but even this argument is open to the objection that the permeability of an unfertilized egg is much less than that of a fertilized one.

Kriszat (1953, 1954) has also investigated the effect of a number of reagents on the rigidity of the cell membrane of sea-urchin eggs. He used the degree of elongation and of stratification of eggs in a centrifuge microscope as measures of the cortical and cytoplasmic rigidity. His results differ from those presented in this paper since he found that glutathione, thioglycollate and periodate decrease the rigidity of the membrane.

SUMMARY

Measurements were made with the cell elastimeter on the effect of a number of reagents and of changes in pH on the rigidity of the cell membrane of unfertilized sea-urchin eggs. The membrane rigidity was increased by dithiopropanol, thioglycollate, chloracetophenone, a polyethanoxide and zinc ions. It was lowered by trypsin, cetyltrimethyl ammonium bromide and sodium dodecyl sulphate. Most of the other reagents, including respiratory inhibitors and calcium ions, had no effect. The membrane has a minimum rigidity at a pH of about 5·7.

I should like to express my thanks to Dr J. H. Schulman, of the Department of Colloid Science, Cambridge, for his kindness in supplying the detergents. I should also like to thank the Director and Staff of the Stazione Zoologica, Naples, for their assistance in this work.

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THE ROLE OF THE SYMBIOTIC BACTERIA IN THE NUTRITION OF *RHODNIUS PROLIXUS* (HEMIPTERA)

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INTRODUCTION

The occurrence of supposedly symbiotic micro-organisms in insects is well known They may be found in the gut, in various other parts of the body and in special organs known as mycetomes. The micro-organisms are usually intracellular, and in many cases are difficult or impossible to grow in artificial culture. They appear to be highly specific to their host and may be transmitted from generation to generation by special and complex mechanisms. Among those previously described are forms considered to be yeasts, bacteria and rickettsiae (Buchner, 1953; Steinhaus, 1940, 1946, 1949; Paillot, 1933).

In some cases, the insect hosts have been shown to be adversely affected by the removal of the micro-organisms and the association is believed to be necessary for the normal development of the host (Brooks & Richards, 1955; Brues & Dunn, 1945; Blewett & Fraenkel, 1944; Brecher & Wigglesworth, 1944).

Blewett & Fraenkel (1944) have produced experimental evidence to show that the yeast-like symbiotes of the two beetles *Stegobium paniceum* and *Lasioderma serricorne* supply vitamins of the B group to their hosts. The symbiotes of the louse, *Pediculus vestimenti*, have a similar function (Puchta, 1955).

In the case of *Rhodnius*, a bacterium occurring in the lumen of the gut has been isolated in artificial culture. It was described as *Actinomyces rhodnii* by Brecher & Wigglesworth (1944), according to the classification of Erikson (1935), and has appeared in the lists of the National Collection of Type Cultures as *Nocardia rhodnii*. Brecher & Wigglesworth also showed that *Rhodnius* nymphs in which the symbiotic bacteria were absent failed to complete their development to the adult stage. Wigglesworth (1936) showed that this organism grown in culture could act as a source of B vitamins for sterile *Lucilia* larvae.

The present paper describes the symbiotic bacteria in the light of recent work on the classification of the filamentous bacteria (Bisset & Moore, 1949; Morris, 1951, 1952), and also provides evidence of the function of these bacteria in the nutrition of the host.

Rhodnius is a member of the Hemiptera and feeds on mammalian blood. There are five nymphal instars in the development from the egg to the adult, and the adult emerges from the fifth and final moult which constitutes metamorphosis.

METHODS

The isolation and examination of the symbiotic bacterium

The adult insects or nymphs were dissected from the dorsal surface and smears were prepared on glass slides from the contents of the midgut; glucose agar plates were inoculated with the same material. The inoculated plates were incubated for up to 7 days at 30° C. Growth of the symbiotic bacteria usually occurred within 3 days. The smears were heat-fixed and stained by Gram's method. It was found unnecessary to sterilize the outside of the insect before dissection because the proportion of contaminated plates when unsterilized insects were used was less than 0·5%.

The rearing of symbiote-free insects

Brecher & Wigglesworth (1944) showed that if the egg surface was sterilized with crystal violet solution the insects which hatched failed to acquire the usual infection with the bacterium. This procedure was adopted, but was discarded when it was found that equally good results could be obtained without sterilization if the eggs were isolated from sources of contamination. The eggs were removed from stocks of mated adults as soon as possible after oviposition and were transferred, in batches of about ten, to sterile tubes. Each insect was transferred to a fresh sterile tube after hatching and thereafter maintained in a sterile environment, being transferred to a fresh sterile tube after each feed and after each moult. Only two insects were found to be infected after such treatment from a total of over five hundred used in the course of the work.

Feeding of the insects

Rabbits and mice were used to provide blood for the feeding of the insects. In the former case, each insect was placed in a sterile test-tube with cotton gauze stretched over the mouth. The tube was inverted upon the ear of the rabbit and the insect was able to feed through the gauze. The mice were anaesthetized with nembutal (Abbots' Laboratories), and the insects in inverted tubes, as described above, were placed on the abdomen. The mice received 0·01 grain (=0·000648 g.) per 100 g. of body weight of nembutal by the intraperitoneal route.

Additions to the blood diet of *Rhodnius* were made by injecting mixed solutions of vitamins intravenously into the rabbit or into the anaesthetized mouse immediately before the insects were fed. The vitamins were prepared in saline solution, stored at −20° C. and mixed immediately before use. The cholesterol content of the rabbits' blood was increased by adding cholesterol to their diet over a period of 2 or 3 weeks before they were required for use.

Rhodnius normally takes one complete feed during each nymphal instar, which results in a moult from which the next instar emerges.

RESULTS

The symbiotic bacteria

The cultures made by the methods described were examined at frequent intervals. Stained films showed that the bacteria were strongly Gram-positive and passed through a life cycle which included filamentous, rod-shaped and coccal forms. The filamentous forms which were multicellular soon fragmented into shorter filaments and rods, and finally into coccal forms consisting of one or two cells. These frequently remained in their original arrangement for some time before dispersing, and gave the appearance of chains of cocci. On transfer to a fresh medium the coccal forms germinated to produce filamentous forms. Branched forms were rare and were transient. In culture the complete cycle lasted about 24 hr. The bacterium produces a salmon pink non-diffusible pigment, which increases in intensity with age and exposure to light. The general morphology and life cycle and the appearance of the colonies confirmed that the bacterium is correctly classified as a member of the genus *Nocardia*, as defined by Bisset & Moore (1949) and Morris (1951, 1952). Further evidence of the systematic position of this organism will be provided in a separate communication elsewhere.

The life cycle of *N. rhodnii* was observed in the insect by the examination of smears made from the gut contents of adults and of nymphs in all stages of development, at daily intervals between successive feeds. The same life cycle occurs within the host insect as that described in artificial culture. The filamentous forms develop within a few days of the insect feeding, they gradually fragment and finally revert to the coccal form. The life cycle in the host occupies from 5 to 8 days. It was found that the bacteria were more numerous in the third, fourth and fifth nymphal instars (that is, at the stages in the insect's life history preceding metamorphosis) than either in the early stage nymphs or in the adult insects. Filamentous forms were rarely found in the adult insects even after they had recently fed. This is of importance in view of the fact, discussed later, that it is these later nymphal instars which are delayed in their development in the absence of the symbiotic bacteria.

Removal of the symbiote and its effect on the development of Rhodnius

Rhodnius nymphs, fed on rabbits' blood, were reared free from *Nocardia rhodnii* by isolating the eggs before hatching and keeping the nymphs in a sterile environment. Their development was compared with that of normal nymphs in which the symbiote was present; the rate of development was measured by the number of days elapsing between the insect feeding and the subsequent moult in each nymphal instar. All the nymphs were kept at 30° C. and the relative humidity was controlled at 75% by means of saturated sodium chloride solution. The development rates are compared in Table 1.

It is seen that in the absence of *N. rhodnii* the first and second instar nymphs developed at approximately the normal rate, whereas in the third instar some nymphs took much longer than normal to moult after feeding. In the fourth instar over

40% of the nymphs which fed failed to moult, and the remainder took up to 30 days more than the normal period before moulting to fifth-instar nymphs. Their further development, after feeding in the fifth and final instar, was arrested, and none of them completed metamorphosis.

Table 1. *The development of symbiote-free* Rhodnius *nymphs compared with that of normal nymphs*

Nymphal instar	*Nocardia rhodnii* absent				*Nocardia rhodnii* present
	No. of insects		No. of days from feed to moult		No. of days from feed to moult
	Fed	Moulted	Average	Range	Range
1	20	17*	8·25	7–10	7–9
2	32	32	9·80	9–12	8–9
3	29	29	18·00	10–51	9–10
4	23	10	28·10	20–40	10–12
5	10	0	—	—	16–20

* The three nymphs which failed to moult died within 2 days of feeding.

When several of the fourth- and fifth-instar nymphs which failed to moult were fed again, moulting occurred in two out of five fourth-instar nymphs and in one out of five fifth-instar nymphs, though only after periods ranging from 39 to 94 days. One of seven nymphs fed for a third time moulted 94 days later.

Moulting was also induced when *N. rhodnii* was introduced into sterile fourth- and fifth-instar nymphs. Infection was produced by transferring these nymphs either to tubes seeded with *N. rhodnii* from cultures, to tubes containing fresh faeces of normal *Rhodnius*; or to stocks of *Rhodnius* nymphs in which *Nocardia rhodnii* was present.

All these nymphs developed normally after they were infected, and the period between infection and moulting was in every case only 2 or 3 days longer than that between feed and moult in normal nymphs at the corresponding stage in development, irrespective of the method of infection, or of the time which had elapsed between feeding and the introduction of *N. rhodnii*.

The next series of experiments was devised to discover if the symbiotic bacteria were supplying some essential B group vitamins to the host insect. Accordingly, symbiote-free nymphs were fed on anaesthetized mice, previously injected by the intravenous route with mixtures of various B vitamins in solution. The concentrations of the vitamins in the various mixtures are shown in Table 2.

Mice were injected with 1 ml. of a mixture for each 10 g. of body weight. The actual quantities of the vitamins injected (μg./g. of blood) are given in Table 3, assuming that the weight of the blood is *c.* 10% of the total body weight of the mouse.

Riboflavin was found to be toxic to mice when injected at the rate of either 100 or 50 μg./g. of blood, and was therefore excluded from the experiments.

Table 2. *Mixtures of B group vitamins for injection into mice*

Vitamin	Source	Final concentration in mixture (mg./ml.)							
		M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8
Thiamine	L. Light and Co. Ltd.	1	0	1	1	1	1	1	1
Nicotinamide	L. Light and Co. Ltd.	1	1	0	1	1	1	1	1
Pyridoxin	L. Light and Co. Ltd.	1	1	1	0	1	1	1	1
Ca pantothenate	Roche Products	1	1	1	1	0	1	1	1
Folic acid	L. Light and Co. Ltd.	1	1	1	1	1	0	1	1
Biotin	L. Light and Co. Ltd.	0·01	0·01	0·01	0·01	0·01	0·01	0	0·01
Cyanocobalamin	Roche Products	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0

The development of the nymphs fed on mouse blood with the addition of various vitamins was observed and compared with that of nymphs fed on mouse blood without additions. Control groups of nymphs with *N. rhodnii* in the gut were also fed on mice and their development compared with that of the symbiote-free nymphs.

Table 3. *Quantities of vitamins added to the mouse blood diet of the nymphs by the injection of the vitamin mixtures*

Vitamin	Quantities added by injection of the mixtures (μg./g. of blood)							
	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8
Thiamine	100	0	100	100	100	100	100	100
Nicotinamide	100	100	0	100	100	100	100	100
Pyridoxin	100	100	100	0	100	100	100	100
Ca pantothenate	100	100	100	100	0	100	100	100
Folic acid	100	100	100	100	100	0	100	100
Biotin	1	1	1	1	1	1	0	1
Cyanocobalamin	1	1	1	1	1	1	1	0

In the first four nymphal instars the nymphs in all the groups developed at the same rate, irrespective of the addition of vitamins or of the presence of *N. rhodnii*; the comparative rates of development in the fifth and final instar are shown in Table 4.

Table 4. *The development of symbiote-free nymphs in the fifth instar with the addition of B group vitamins to the mouse blood diet*

Vitamin supplement	No. of insects		No. of days from feed to moult		
	Fed	Moulted	Average	Range	
M-1	7	6	20	19–22	*N. rhodnii* absent
M-2	6	5	21·5	16–26	
M-3	7	7	22	19–27	
M-4	5	4	29	21–33	
M-5	7	6	23	18–28	
M-6	6	6	22	19–27	
M-7	7	7	19·5	19–20	
M-8	6	4	19·5	19–21	
None	7	4	47	27–68	
None	5	5	17·5	16–19	*N. rhodnii* present

When all the vitamins used in the experiment were added simultaneously to mouse blood, all but one of the nymphs fed on the supplemented diet (M-1) were enabled to complete their development. Furthermore, those nymphs which moulted after feeding did so after approximately the same period of time as normal nymphs in which the symbiote was present. The omission from the mixture of either thiamine (M-2), pyridoxin (M-4), Ca pantothenate (M-5) or cyanocobalamin (M-8) resulted in one or two nymphs in each case failing to moult, but when either nicotinamide (M-3), folic acid (M-6) or biotin (M-7) was omitted all the nymphs moulted. The nymphs fed on blood from which biotin (M-7) or cyanocobalamin (M-8) was omitted moulted after approximately the same interval as did normal nymphs, but in the other groups of nymphs some took longer to moult. This delay in moulting was most pronounced when pyridoxin was omitted, and less so when Ca pantothenate, nicotinamide, folic acid or thiamine was omitted. The nymphs which received no vitamin supplement took a very much longer time than normal nymphs to moult after they were fed on mouse blood, and a high proportion of them failed to moult. Similar symbiote-free fifth-instar nymphs fed on rabbits' blood always failed to moult (Table 1) and the period between feed and moult in the third and fourth instars was also longer than that in normal nymphs. This fact, and the increase in the development rate brought about by the addition of B group vitamins to mouse blood, suggested that the difference in the development rate of nymphs fed on mouse blood on the one hand and on rabbit blood on the other may be due to a higher concentration of some of the B vitamins in normal mouse blood. Symbiote-free nymphs were therefore fed on rabbits previously injected with a mixture of the B group vitamins and their development was compared with that of similar nymphs fed on normal rabbits. Riboflavin was again omitted from the mixture because of its toxicity at the desired concentration. Inositol (L. Light and Co. Ltd.) and choline (L. Light and Co. Ltd.) were included in these experiments. The rates of development of all these nymphs are shown in Table 5.

The addition of the vitamins caused an increase in the rate of development of the fourth-instar symbiote-free nymphs fed on rabbit blood, but none of them completed their development to the adult stage. Furthermore, these nymphs developed less rapidly than similar nymphs fed on mouse blood without vitamin supplement, even though the concentration of the added vitamins was undoubtedly greater in the supplemented rabbit blood than in the normal mouse blood. The addition of inositol and choline chloride had little effect on the rate of development. It therefore appears that some other factor is supplied by *N. rhodnii* to its host, that this factor is essential to the normal development of *Rhodnius* and is present in a greater concentration in mouse blood than in rabbit blood.

This factor could not be identified with cholesterol because symbiote-free nymphs fed on rabbits with a high cholesterol level in their blood developed no more rapidly than those on normal rabbits, and less rapidly than those on normal mice. The cholesterol levels were: normal rabbit, *c.* 750 μg./g. of blood; high cholesterol rabbit, *c.* 3000 μg./g. of blood; normal mice, *c.* 1000 μg./g. of blood.

Table 5. *The development of symbiote-free* Rhodnius *nymphs fed on rabbit blood supplemented with B group vitamins*

Nymphal instar	Vitamin* supplement	No. of insects		No. of days from feed to moult	
		Fed	Moulted	Average	Range
3	R-1	9	9	11·5	11–13
	R-2	1	1	13	13
	R-3	1	1	11	11
	None	4	4	14	11–16
4	R-1	9	9	20	12–31
	R-2	4	4	14	10–18
	R-3	3	3	19	13–27
	None	9	1	17	17
5	R-1	9	0	—	—
	R-2	6	0	—	—
	R-3	3	0	—	—
	None	1	0	—	—

* Vitamin supplements:
R-1 contained thiamine, nicotinamide, pyridoxin, Ca pantothenate, folic acid, biotin and cyanocobalamin but no inositol or choline chloride.
R-2 contained all the above vitamins and inositol (final concentration = *c.* 500 g./g. of blood).
R-3 contained all the above vitamins and inositol and choline chloride (500 g./g. of blood).

CONCLUSIONS

The results presented in the first part of this paper confirm those of Brecher & Wigglesworth (1944) who found that the bacterium is extracellular, in the gut lumen, and no special mechanism exists for the transfer of the bacterium from generation to generation of the host. *Nocardia rhodnii* is transmitted in the faeces of the host, and the infection is acquired from the egg surface only when it is contaminated by excrement from infected nymphs and adults. In their natural environment these insects are gregarious, living in the burrows of the small mammals on which they feed and hiding in crevices in the burrows between feeds. Under these conditions the method of transmission of the bacterium is undoubtedly effective, because of the close contact of the insects for long periods. However, laboratory cultures of the insects may at times die out because of the failure of the transmission mechanism, particularly if the insects are kept in small numbers and under relatively clean conditions. *N. rhodnii* is therefore essential to the normal development of the host insect, and in its absence the insect fails to reach the adult stage.

The evidence presented in the second part of the present work shows that the function of the bacterium is the supply of certain B vitamins in which the normal blood diet of *Rhodnius* is apparently deficient. The insect is dependent on the symbiotic bacteria for its supply of pyridoxin, Ca pantothenate, nicotinamide and thiamine when it is fed on mouse blood. Biotin and folic acid are, however, present in adequate concentration in the blood to meet the insect's requirements. The position of cyanocobalamin is not clear because the results were somewhat conflicting, one-third of the nymphs tested appeared to be dependent on the bacterium

for the supply of this vitamin whereas the rest obtained adequate quantities for their normal development from mouse blood. Another factor is also required by *Rhodnius* for normal development and is normally supplied by the bacterium. This has not been identified but is present in greater concentration in mouse blood than in rabbit's blood. It cannot be identified with any of the B vitamins investigated, nor with choline chloride, inositol or cholesterol because rabbit's blood with the addition of any or all of these factors was less adequate as a diet for the symbiote-free *Rhodnius* nymphs than normal mouse blood, even though the supplemented rabbit's blood contained greater concentrations of the vitamins than the normal mouse blood. *Rhodnius* develops equally well and at the same rate on the blood of either species when *Nocardia rhodnii* is present, proving that the unidentified factor is supplied by the bacterium. Riboflavin had to be excluded from these experiments because it was not tolerated by mice or rabbits at the dosage level required, and it is possible that it could be the unidentified factor. Variations in the experiments such as that mentioned in connexion with cyanocobalamin may be caused by the limiting influence of the unidentified factor. Variations of this kind are to be expected when it is realized that the blood used in these experiments as the basic diet was not, of course, free from vitamins, and furthermore the concentrations of the vitamins would vary considerably in different animal species, in different individuals of the same species and in an individual at different times.

Further work on the identification of the unknown factor is in progress, and it is hoped to clarify the results further by the use of a basic diet which can be standardized and fed to the insects through a membrane. However, it is clear that the role of the symbiotic bacterium in *Rhodnius* is similar to that of other symbiotic micro-organisms in other insect species so far studied.

Symbiote-free *Rhodnius* nymphs are bacteriologically sterile, and it appears possible that they could be usefully employed in the investigation of certain nutritional problems which are normally complicated by the presence of bacteria in the gut of the experimental animals employed.

SUMMARY

1. Earlier views of the nature of the association between *Rhodnius* and its symbiotic bacteria are confirmed.

2. The bacterium is described as *Nocardia rhodnii* in accordance with more recent views of the classification of the Gram-positive filamentous bacteria.

3. Experimental evidence is provided to show that the symbiotic bacterium is essential to *Rhodnius* because of its function in supplying certain B group vitamins, in which the normal blood diet of the host is deficient.

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CELLULOSE DIGESTION BY THE SILVERFISH *CTENOLEPISMA LINEATA**

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It is the prevailing opinion to-day that the majority of species of animals which feed upon plants or plant products are unable to hydrolyse cellulose in spite of its abundance (Baldwin, 1952), depending instead upon the sugars, starches, fats and proteins present in the plant tissues. This implies the lack of a suitable alimentary enzyme, cellulase, or symbiotic gut micro-organisms which could perform that function. Study of cellulose digestion in animals is hampered by the difficulty of defining the role of the micro-organisms of the gut. If cellulolytic action is found in extract of tissues or in secretions of glands, the question remains whether or not this activity is attributable to micro-organisms; conversely, finding a cellulose-digesting microbe in the intestine of an animal does not prove conclusively its benefit to the animal.

Although observations on the feeding habits of the silverfish have been numerous and date back to Hooke's *Micrographia* (1665), its nutrition has been little investigated. However, Lindsay (1940) found the silverfish *Ctenolepisma longicaudata* would eat any kind of cellulose but preferably the most degraded. He also found that a diet of cellulose alone kept the animal alive for a longer time than if it were starved. He reported obtaining an enrichment culture of cellulose-digesting bacteria from the crop of a single animal, and from this concluded that digestion of cellulose in silverfish is accomplished by symbiotic micro-organisms of the gut.

The object of the present investigation was to define more clearly whether silverfish digest and metabolize cellulose and whether this is accomplished by symbiotic micro-organisms or by enzymes secreted from the cells of the gut. The silverfish *Ctenolepisma lineata* was chosen for this purpose because specimens are easily obtained in numbers on the bark of local *Eucalyptus* trees. They thrive in the laboratory at 25° C. and at a relative humidity of 80%. They were fed on a diet of rolled oats in darkened gallon jars containing a nidus of absorbent cotton. Breeding takes place usually in April and May, at which time the eggs are laid. Young could be grown to maturity under laboratory conditions without difficulty; therefore they served admirably for some of the experiments spanning a long time and involving tests of growth and moulting.

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DEMONSTRATION OF CELLULOSE DIGESTION IN THE SILVERFISH

By determining the respiratory quotient (R.Q.) of silverfish, fed for a month on a cellulose diet (Whatman no. 43 filter-paper), it should be possible to gain indicative evidence of cellulose digestion. For this study three or four silverfish were placed into each of a number of small Warburg flasks attached to small-bore manometers and the gaseous exchange was followed for a day. Readings were taken after the animals had become acclimatized to the prevailing temperature of the water-bath (27·2° C.) and the confined area of the flasks at which time they became relatively quiet and remained so for the remainder of the experiment.

The R.Q. on cellulose was 0·91, 1·09 and 0·92 in a series of three experiments, whereas silverfish, fed for a month on gelatin (Gelfoam) and tested in a similar manner to those on cellulose, gave an R.Q. of 0·75. The results suggest that cellulose-fed animals are metabolizing almost pure carbohydrate.

More definite evidence for digestion of cellulose was obtained by feeding individual silverfish pure cellulose, recovering the faecal pellets as they were dropped, and determining the digestibility coefficient (Roeder, 1953) for the silverfish by the following ratio:

$$\% \text{ digestibility} = \frac{\text{Dry weight of food consumed} - \text{dry weight of excrement}}{\text{Dry weight of food consumed}} \times 100.$$

When digestion is complete no excrement is obtained and the coefficient is 100%. Some typical values for insects include 24% for the silkworm larva, 48·5% for the armyworm, *Prodenia eridania*, and 46·3% for the mealworm, *Tenebrio molitor* (Roeder, 1953). From the data in Table 1 for silverfish fed on cellulose for a month or more, it is evident that the silverfish digests more of the food taken in than the other insects listed above, suggesting that it digests cellulose while the phytophagous insects do not. In this respect it resembles the dairy cow with a coefficient of 72% for dried grass (Maynard, 1937). Not only did the silverfish digest a considerable amount of the cellulose eaten but in many cases they gained weight even when fed on cellulose alone.

The most decisive evidence of cellulose digestion was obtained by feeding silverfish with uniformly labelled ^{14}C cellulose. In these experiments a well-fed adult silverfish (*c.* 15–25 mg.) was put into a standard, single-arm Warburg vessel without a centre well, fitted with a standard taper hollow glass tube which was torch-sealed at the untapered end. A sample of radioactive cellulose weighing several milligrams was included as food for the animal, and the side arm was filled with 0·4 ml. freshly prepared 5% sodium hydroxide. After a month or longer the alkali, which had absorbed the carbon dioxide produced during respiration, was removed and transferred to a conical 12 ml. centrifuge tube to which 6% barium chloride was added dropwise until no further precipitation of barium carbonate was observed. The tube was then centrifuged, the residual alkali decanted, and the precipitate washed several times with 95% ethyl alcohol. The control without an animal served as a blank for carbon dioxide absorbed from the air. The barium carbonate

was spread on aluminum planchets (Calvin *et. al.* 1949) and the radioactivity was measured by a helium-flow, windowless Geiger counter (Tracerlab Autoscaler). The results of these tests are given in Table 2. The high radioactivity of the carbon dioxide respired by the silverfish indicates extensive metabolism of nutrient derived from radioactive cellulose which in turn could happen only if the silverfish had digested cellulose since it was the only food available.

Table 1. *Digestibility coefficients for silverfish fed cellulose for several months*

No.	Initial wet wt. silverfish (mg.)	Dry wt. cellulose consumed (mg.)	Dry wt. of faeces (mg.)	Digestibility coefficient (%)
1	22·21	9·39	1·37	85·4
2	25·21	7·46	1·94	74·2
3	25·38	3·35	0·99	73·2
4	20·01	1·58	0·38	75·9
5	23·51	4·49	0·58	87·0
6	27·25	2·33	0·63	71·7
7	15·67	5·99	0·84	85·8

Table 2. *Radioactivity of* $Ba^{14}CO_3$ *derived from the respiratory* CO_2 *of silverfish fed* ^{14}C *cellulose*

Planchet no.	mg. $BaCO_3$	Counts* per min.	*d*/min./mg. $BaCO_3$	*d*/min./0·1 mM $BaCO_3$
		Experiment 1		
1	4·0	3710	3840	$7·58 \times 10^4$
2	6·5	5150	3380	$6·69 \times 10^4$
3	1·3	1100	3230	$6·37 \times 10^4$
4	2·0	1730	3420	$6·75 \times 10^4$
5	2·1	1750	3300	$6·51 \times 10^4$
6 Background	—	59	—	—
		Experiment 2		
1	2·9	1510	2560	$5·05 \times 10^4$
2	2·0	1200	2830	$5·58 \times 10^4$
3	1·2	855	3220	$6·36 \times 10^4$
4 Background	—	45	—	—

* Counts per minute are presented after correction for self-absorption, coincidence and the efficiency of the counter which was determined in each run by counting a radioactive standard sample; *d* = disintegrations.

Additional evidence for incorporation of radioactive cellulose came from determination of radioactivity of the tissues of the silverfish. For this purpose silverfish were fixed in formol-acetic-alcohol, dehydrated in alcohol, embedded in paraffin and sectioned in the sagittal plane. The paraffin was first removed from the 100 μ thick sections, and then from the resulting dried tissue remnants of the gut were removed under a dissecting microscope, leaving only muscle and glandular tissue. The remaining section, placed under a Geiger counter, gave 1400 counts per

0·46 mg. dry tissue per minute, a count equivalent to 3120 disintegrations per mg. of dry silverfish tissue without the intestine or its contents. This value is many times the background. Incorporation of radioactive carbon could have occurred only if the cellulose had been digested.

LACK OF CELLULOLYTIC ACTION OF SILVERFISH GUT MICRO-ORGANISMS

Since the silverfish used in the experiments described in the first section carry a population of micro-organisms in various parts of the gut, especially in the crop, it was necessary to determine whether the microflora might account for cellulose digestion. When a microfauna exists it consists only of large colourless parasitic sporozoans averaging about fifteen per animal and could be excluded from consideration on this account.

An attempt was therefore made to determine whether cellulolytic microbes occurred in the gut. For this purpose into each of twenty sterile 125 ml. Erlenmeyer flasks containing 75 ml. of Hungate's mineral medium (1950) and containing folded pieces of Whatman's no. 43 filter-paper as a source of cellulose was placed the entire gut of a silverfish, with aseptic precautions. The flasks were incubated at room temperature for over a month. Only seven showed cellulose decomposition, and in these moulds, not bacteria, were responsible. Since moulds were never seen growing in the gut of the silverfish, the best explanation of the results is that during their extensive grazing the silverfish had eaten spores of moulds growing on wood.

Since the silverfish gut contains nutrients other than cellulose, it was possible that micro-organisms might actively hydrolyse cellulose only in the presence of other nutrients. Therefore, forty additional tests were made in which the cellulose was supplemented with protein, carbohydrates, nucleic acid hydrolysate and vitamins in addition to minerals. Various combinations and concentrations, as suggested by demands of various micro-organisms but particularly those digesting cellulose, were tested. Although many bacteria developed in such media they did not decompose cellulose.

Since it was necessary also to exclude the possibility that anaerobic bacteria might decompose the cellulose in the gut of the silverfish, twenty-six tests were made following methods devised by Hungate (1950) for detection of anaerobic cellulolytic micro-organisms. These tests were negative, and it became evident that anaerobic conditions were unfavourable for the silverfish microflora, since only small populations developed under anaerobic conditions, yet when oxygen was admitted to the anaerobic test plates many additional colonies appeared.

It must be admitted that negative results are never as convincing as positive ones, and that even the numerous media tested may not have provided favourable conditions for growth of cellulolytic bacteria. Perhaps the enriching nutrients, even in a mash of silverfish intestine, are inhibitory to cellulolytic bacteria. But since so few bacteria are normally seen in the gut, and since no unequivocal evidence of cellulose digestion by gut bacteria was observed, this line of attack was discontinued as unprofitable.

Finally, it is possible that intracellular symbionts might serve to digest cellulose. Such agents have been implicated in insect digestion by Buchner (1953) and culture of some forms has been claimed (Glaser, 1930); the method available to check this possibility is to examine sections of the gut for mycetomes, mycetocytes or other structures similar in nature to those found in other insects. Examination of 10 μ sections at various levels of the gut of the silverfish stained in various ways, such as haematoxylin and eosin, Gram's stain, van Gieson's stain and safranin and fast green, considered diagnostic (Brooks & Richards, 1955), revealed no such structures. The gut of the silverfish is relatively simple in structure compared to that of other insects, consisting of a thin-walled oesophagus and crop, a proventriculus with well-developed teeth, and a glandular midgut which leads into a smooth-walled hindgut and rectum. If intracellular symbionts exist they escape detection by the accepted methods. In passing it may also be pointed out here that histological study shows no 'fermentative chambers' or proctodaeal pouches where extra-cellular micro-organisms might be harboured.

DIGESTION OF CELLULOSE IN BACTERIA-FREE SILVERFISH

If the silverfish does not depend upon micro-organisms to digest its cellulose, digestion should be possible in the complete absence of micro-organisms. For this purpose it is necessary to obtain silverfish in bacteria-free culture on a synthetic medium. It proved possible to sterilize eggs and to raise the silverfish on sterile nutrients. Eggs obtained during the April and May breeding season were sterilized by washes in White's solution (1931) containing mercuric chloride and alcohol. They were picked up individually with a damp inoculating loop which had previously been flamed and cooled in sterile distilled water. They were transferred to the first depression in a sterile agglutination dish containing 0·2 ml. of White's solution. They were then successively transferred to three depressions containing a like amount of sterile distilled water, care being taken to transfer a minimum of liquid in each case so as to dilute the first solution maximally. They were then transferred to a sterile test-tube containing a piece of sterile, dried, rolled oats which had previously been soaked in a solution containing 1% yeast extract and 0·02% liver extract concentrate. The small amount of water still clinging to the eggs was absorbed by the piece of oat.

That this procedure sterilizes the eggs was demonstrated by plunging a sample of eggs treated as above into vessels containing sterile Bacto A-C medium (Difco Laboratories, 1948) which supports a comprehensive list of anaerobic and aerobic organisms and is widely used to test sterility. In no case did micro-organisms appear, and nymphs hatched out in the medium when eggs were not crushed (some eggs were crushed to test for micro-organisms which might not be able to get through the egg membrane). Also, cultures containing 4 mm. scaled juveniles which had been grown on sterile media from washed eggs showed no evidence of micro-organisms. On the other hand, media inoculated with eggs taken directly from the field or from ordinary laboratory cultures showed copious growth of

micro-organisms of a wide variety. The method may therefore be considered satisfactory.

Sterile nymphs which had been allowed to grow to 4 mm. in length on sterile oats enriched by soaking in yeast extract and liver extract were placed in the sterile Warburg vessels such as were described in the first section. In one set of experiments only ^{14}C cellulose was used, in the second ^{14}C cellulose and oats because silverfish do not live well on cellulose alone. Aseptic precautions were followed throughout the experiment. The results are given in Table 3.

Table 3. *Radioactivity of* $Ba^{14}CO_3$ *derived from the respiratory carbon dioxide of bacteria-free silverfish**

Animal no. 1, fed ^{14}C cellulose with no additional food; nos. 2 and 3, fed ^{14}C cellulose and oats soaked in yeast extract and liver extract.

Planchet no.	mg. $BaCO_3$	Counts per min.	*d*/min./mg. $BaCO_3$	*d*/min./0·1 mM $BaCO_3$
		Animal no. 1		
1	1·7	458	1480	$2{\cdot}92 \times 10^4$
2	1·2	600	2710	$5{\cdot}34 \times 10^4$
3	0·6	277	2360	$4{\cdot}65 \times 10^4$
4	0·5	379	3600	$7{\cdot}10 \times 10^4$
5 Back-ground	—	33	—	—
			Average	$5{\cdot}12 \times 10^4$
		Animal no. 2		
1	1·9	255	604	$1{\cdot}19 \times 10^4$
2	2·8	384	627	$1{\cdot}23 \times 10^4$
3	0·3	74	740	$1{\cdot}46 \times 10^4$
4 Back-ground	—	38	—	—
			Average	$1{\cdot}29 \times 10^4$
		Animal no. 3		
1	2·5	164	327	$6{\cdot}44 \times 10^3$
2	1·8	134	342	$6{\cdot}73 \times 10^3$
3	0·5	68	446	$8{\cdot}78 \times 10^3$
4	0·3	52	456	$8{\cdot}97 \times 10^3$
5 Back-ground	—	29	—	—
			Average	$7{\cdot}73 \times 10^3$

* The animals weighed less than 1 mg. each.

The data clearly indicate that bacteria-free silverfish produce as much carbon dioxide with ^{14}C as do the animals taken directly from laboratory cultures when fed cellulose alone. When fed cellulose and oats, less carbon dioxide with ^{14}C appears, undoubtedly because the animals graze more on the oats and eat less cellulose. The data offer conclusive proof that the silverfish possesses a cellulase.

CELLULOLYTIC ACTIVITY OF GUT EXTRACTS; OTHER CARBOHYDRASES IN THE GUT

Since the bacteria-free silverfish digests cellulose it should be possible to detect a cellulase from its gut. Preliminary tests were made with each of the digestive organs of the silverfish in turn: crop, salivary gland and midgut. Negative results were obtained with all but the midgut. Histological examinations indicate that of all the

digestive organs only the midgut has a secretory epithelial lining with goblet-type secretory cells. Subsequent work was therefore confined to the midgut.

The midgut of each of twenty silverfish was removed from animals narcotized with carbon dioxide, by pinching with a pair of watchmaker's forceps at the fourth or fifth abdominal segment and gently teasing the animal apart with a second pair of forceps. This exposed the gut chiefly at the point of juncture between midgut and proventriculus. The midgut was grasped behind the main lobes and teased away from the rest of the intestine. The tissue was ground in a Potter homogenizer chilled with ice water, (Dockstader & Halvorson, 1950) the tip of the grinding rod being dipped into 0·02 M-phosphate or citrate buffer and placed in the tube. As each midgut was dissected it was immediately placed in the homogenizer and ground with a gentle turning motion. Since silverfish tissues are exceptionally soft, no abrasive was necessary and microscopical examination disclosed few intact cells. After the last midgut had been ground the grinding rod was washed by dropping cold buffer on it over a 12 ml. graduated conical centrifuge tube into which the contents of the homogenizer were rinsed with several washings of cold buffer until a total of 2–3 ml. of homogenate suspension had been collected. The contents were centrifuged and only the decanted supernatant liquid was used for enzyme determinations.

To test the extract for cellulose digestion, to 0·2 ml. of the enzyme solution in buffer contained in a centrifuge tube was added a 0·1 ml. sample of regenerated cellulose suspension (Trager, 1932) in 0·02 M-phosphate buffer. A layer of toluene was added and the contents of the tube were allowed to incubate overnight at room temperature. Controls included substrate solution alone with buffer, and enzyme solution alone with buffer. An aliquot (0·1 ml.) was removed at the beginning of the experiment from each tube and the protein precipitated by adding 0·1 ml. each of the barium hydroxide and zinc sulphate solutions of Somogyi (1945); after 24 hr. similar aliquots were again removed and tested. In each case the tubes were centrifuged and the supernatant liquid was tested for reducing sugar by the method of Somogyi (1952).

For measuring sugar a standard curve was first obtained at 530 $m\mu$ with known amounts of sugar using the Beckman spectrophotometer modifier for small samples with pinhole slit for micro-cuvettes. Sugar present in test samples could then be determined by directly reading the sugar concentration corresponding to the optical density on the standard curve.

The data in Table 4 indicate that the midgut extract contains a cellulase. Its activity is less than that of the cellobiase and the amylase which were tested in a similar manner except that the initial dilution of homogenate was double, and twice the amount of various reagents was added in making the tests.

The activity of the cellulase depends upon the pH, and in Fig. 1 the pH/activity curve is plotted. The experiments were performed in the same manner as described above, buffer at an appropriate pH being used in the dilutions. The activity maxima were about half a pH unit apart in two successive tests; this was not the result of a pH change, as checks indicated constancy over the period of experimentation.

Table 4. *Determination of an amylase, cellobiase and cellulase from the midgut of the silverfish*

Substrates: soluble starch, 1 %; cellobiose, 0·01 %; regenerated cellulose, 0·03 %. Phosphate buffer (0·02 M) pH 6·7 for amylase and cellobiase, and McIlvaine's buffer (0·025 M) pH 4·7 for cellulase.

Reaction mixture	Time of sampling (hr.)	Reducing sugar at time of sampling (μg.)	Increase in sugar after 24 hr. (μg.)
Midgut + buffer	0	1·0	—
Midgut + buffer	24	1·5	0·5
Starch + buffer	0	1·0	—
Starch + buffer	24	2·0	1·0
Midgut + starch	0	22·0*	—
Midgut + starch	24	300·0	278·0
Cellobiose + buffer	0	20·0	—
Cellobiose + buffer	24	20·0	0
Cellobiose + midgut	0	20·0	—
Cellobiose + midgut	24	95·0	75·0
Cellulose + buffer	0	Trace	—
Cellulose + buffer	24	Trace	0
Cellulose + midgut	0	Trace	—
Cellulose + midgut	24	15·0	15·0

* This large quantity of sugar produced at 'zero hours' is attributable to hydrolysis occurring in the period of time (*c.* 15 min.) which elapsed after addition of substrate to enzyme mixture before protein precipitation.

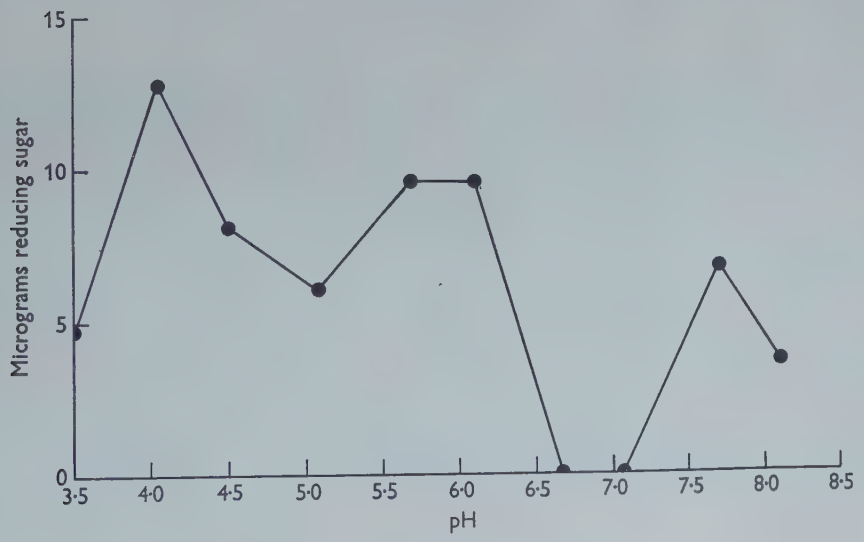

Fig. 1. The pH activity curve of silverfish cellulase.

The pH/activity curve for cellobiase was also determined and is given in Fig. 2. This enzyme attacks the cellobiose molecules which are formed by the action of cellulase upon the cellulose and is therefore of interest in this connexion.

In the final series of experiments the cellulase was further characterized by determining its precipitation with ammonium sulphate. For these experiments twice as many midguts were used as in the above experiments, and the centrifugate was reground to extract as much of the enzyme as possible. The initial solution of

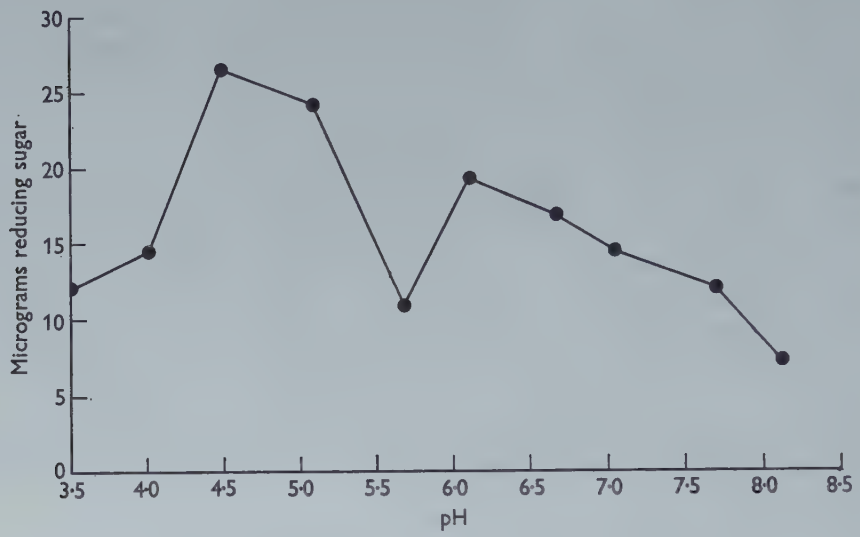

Fig. 2. The pH activity curve of silverfish cellobiase.

enzyme from forty silverfish amounted to 3 ml. To this was added the appropriate volume of saturated ammonium sulphate (which had been brought to pH 6·7 by dropwise addition of 10% potassium hydroxide), until 50–60% saturation had been reached. After this had been centrifuged, more saturated ammonium sulphate was added to the decanted supernatant fluid to bring it to 80% saturation and the procedure was repeated. For 100% saturation, crystals of ammonium sulphate were added. The high-speed Servall angle centrifuge was used to centrifuge the sample, except in initial experiments. In one series of experiments ammonium sulphate was added to test 10% intervals. No precipitate formed until 50% saturation was reached; at 50% heavy precipitation occurred and 60, 70 and 80% produced precipitates. Each centrifugate was dissolved in distilled water and washed separately into individual tubes of Visking dialysis tubing ($\frac{8}{32}$ in. diameter) in which it was dialysed against distilled water overnight with frequent changes of distilled water.

To test the activity, each sample was first dissolved in distilled water, dialysed, then buffer was added to give an optimal pH for cellulase and cellobiase activity. The data are given in Table 5. Cellulase activity is concentrated in the 60, 70 and some cases the 80% fractions.

Table 5. *Separation of cellulase activity of soluble extracts of silverfish midgut into fractions by ammonium sulphate precipitation*

Exp. no.	Fraction (%)	μg. reducing sugar produced per 1 ml. of enzyme solution per 24 hr. period
1	50–60	39·5 Adams centrifugate
	80	7·2 Adams centrifugate
	100	25·2 Servall centrifugate
2	50–60	15·8 Servall centrifugate
	80	17·3 Servall centrifugate
3	50	Trace Servall centrifugate
	60	20·0 Servall centrifugate
	70	30·0 Servall centrifugate
	80	Trace Servall centrifugate

DISCUSSION

The experiments described above clearly show that the silverfish digests cellulose and that it does so by virtue of its own enzymes, not by a cellulolytic flora or fauna. Other animals have been considered capable of cellulose digestion, notably some protozoans (Hungate, 1942, 1946), *Helix* (Holden & Tracey, 1950), *Teredo* (Greenfield & Lane, 1953), and some insects (Ripper, 1930; Mansour & Mansour-Bek, 1934). At the present time some question exists concerning animal cellulases because bacteria-free animals were never used. Some of these experiments should be repeated using tracer techniques on bacteria-free animals.

Perhaps the most successful animals which use cellulose as food depend upon a gut microflora, like the ruminants (Hungate, 1950) or upon a microfauna like many of the termites (Cleveland, 1924). This suggests that Nature's experiments with animals producing their own cellulases have been less successful during the course of evolution than her experiments with cellulolytic symbionts. In the case of ruminants, the capacity of bacteria to incorporate inorganic nitrogen in synthesizing protein, a process foreign to animal tissue, gives the symbiotic method considerable advantage over simple digestion of cellulose. The advantage in the termite, where this does not occur, is less clear. The silverfish has achieved a unique niche by virtue of its ability to utilize cellulose, since this removes it from active competition with more aggressive and highly differentiated insects. Experiments clearly indicate that silverfish cannot live on cellulose alone, since they invariably die in about a month on such a diet. However, growth occurs when a nitrogen source such as oat is supplied. This subject is the topic of a second study which will be reported separately.

SUMMARY

1. The silverfish, *Ctenolepisma lineata*, on a diet of cellulose alone shows a respiratory quotient of close to unity, indicating utilization of carbohydrate, presumably derived from cellulose.

2. The silverfish may gain weight temporarily on a diet of cellulose alone although the diet is not satisfactory for prolonged feeding.

3. The silverfish digests part of the cellulose ingested, the utilization efficiency being comparable to that of the dairy cow.

4. Silverfish fed cellulose uniformly marked with ^{14}C respire $^{14}CO_2$, indicating that cellulose is metabolized and therefore must have been digested.

5. The gut of the silverfish contains many micro-organisms, but none of the bacteria grown in favourable culture media are capable of digesting cellulose. A few moulds do, but they are never seen growing in the gut and are presumably developed from spores grazed from wood by the silverfish.

6. Bacteria-free silverfish were obtained by washing eggs in a solution of mercuric chloride and ethanol and raising the nymphs on rolled oats and vitamins under aseptic conditions.

7. Bacteria-free silverfish fed cellulose uniformly marked with ^{14}C respire $^{14}CO_2$, indicating that even in the absence of micro-organisms, *C. lineata* metabolizes cellulose and therefore must have digested it.

8. A cellulase was demonstrated in extracts of the midgut. A cellobiase and an amylase were also shown to be present. The pH optima for the cellulase are 4·0 and 6·0, with a smaller peak occasionally showing at 7·7. For cellobiase the optima were 4·5 and 6·5.

9. The cellulase was isolated in the 60 and 70% ammonium sulphate saturated fractions of the soluble proteins from the midgut.

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SUN NAVIGATION OF *APIS MELLIFICA* L. IN THE SOUTHERN HEMISPHERE

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INTRODUCTION

Honey bees can orientate by means of the sun, taking the hour of the day into account (v. Frisch, 1952); colonies containing workers which had been trained during a sunny evening to search for food in a particular geographical direction were transferred overnight to a locality unknown to them; during the following sunny morning the trained bees searched predominantly in the 'learned direction', in spite of the fact that during the training in the evening the sun had been in the west, while during observation on the following morning it was in the east. This faculty of recognizing the geographical direction, which bees share with some other arthropods and with birds (see Kalmus, 1954), can be explained by assuming the working of an internal mechanism or reference system which compensates for the daily movements of the sun's azimuth. In temperate northern regions, where v. Frisch's observations were made, the daily course of the sun is clockwise; it seemed interesting to study the behaviour of honey bees trained in a similar way in a region of the southern hemisphere, where the sun moves counter-clockwise.

Investigations were carried out on bees whose ancestors had lived for several centuries in the southern hemisphere and on bees which were of recent northern hemisphere origin and whose parents had been transferred to the southern hemisphere.

MATERIAL AND METHODS

The experiments were performed during the south Brazilian winter in May and June 1955, when fairly dry weather, the paucity of bee crops and the low elevation of the sun at noon offered suitable conditions. The region was that of Piracicaba (22° 40′ S.) in the state of São Paulo; there vast areas planted with sugar cane as well as fallow and orchards provided numerous sites poor in landmarks, and the existence of a well-equipped bee department in an agricultural college made the organization of the work possible.

The following types of colonies were used in the investigation:

(1) Black bees belonging to a local bee keeper; these were said to be mainly descended from European colonies imported from Portugal during the last two to four centuries, with some possible admixture of later imports also from Europe.

(2) Locally reared light offspring from 'Italian Queens' bred and inseminated in California and brought to a Brazilian apiary. (No workers actually reared in the northern hemisphere and transferred to Brazil were available.)

(3) 'Hybrid' bees, descendants from 'Italian queens' imported during the last decade from California into Brazil, showing by their coloration various degrees of genetical admixture with local black drones.

The existence of a mechanism compensating for the daily changes of the sun's direction has been demonstrated in two different ways as described by v. Frisch (1952):

(1) By observing a gradual change in the direction of dance of a few accidentally observed bees which for unknown reasons continued to dance for an hour or more instead of for only a few minutes.

(2) By shifting colonies containing workers trained during the evening to search for food in a particular direction, and by observing the direction in which they search on the following morning. Only this second method was used in the present work; details can be gathered from the following descriptions of experiments.

RESULTS

The results of five experiments are described in some detail and all other results are summarized.

A. *Black Brazilian honey bees*

In the evening of 6 June, hive no. LA containing a flourishing colony of local black bees was put in the middle of a large flat field covered with about 1 ft. high sugar cane in rows 1 m. apart, running in an approximately north-south direction. The sugar cane in adjacent fields was higher; no larger trees were anywhere nearer than a mile. In the morning of 7 June, which was a sunny day throughout, the hive entrance (which was facing north-west) was opened, and at noon a dish of scented syrup was put on each of three wooden stands near it. Feeding bees were observed after 1½ hr. The stands were then gradually moved away from the hive in a direction of 15° E. of N.; a distance of 140 m. was reached at 16.30 hr. Two stands were now removed, leaving only one. The initially small number of visitors now increased rapidly so that it was possible to mark 217 bees between 16.43 hr. and sunset at about 17.36 hr. Half an hour later the hive entrance was blocked and the colony was moved to a young orchard some 5 km. away. There were no rows of sugar cane and higher trees were entirely absent.

Early in the morning of 8 June, four stands with scented syrup dishes similar to those used on the previous day were placed at a distance of 140 m. from the colony in the following directions: 15° E. of N., 15° S. of E., 15° W. of S., 15° N. of W. Observers were posted to kill and record all honey-bee visitors to these dishes, and at 8.38 hr. the hive entrance, now facing north-east, was unblocked. Apart from some haze during the first 2 hr. of observation the weather was sunny and only an occasional breeze from the north interrupted the quiet air. Fig. 1 shows the visits of marked and unmarked bees which were caught on the four dishes during the day. Only one visitor escaped, and it did not return. Bees which circled in the neighbourhood of the observers but did not settle were noted but are not included in the figure.

Fig. 1 indicates that at all times most bees were caught at the dish in the direction to which they had been trained. The results of this and of three similar experiments with black local honey bees trained in different geographical directions are summarized in Table 1; in the four experiments 81, 76, 33 and 50% of the bees were caught before noon at the geographical direction of training.

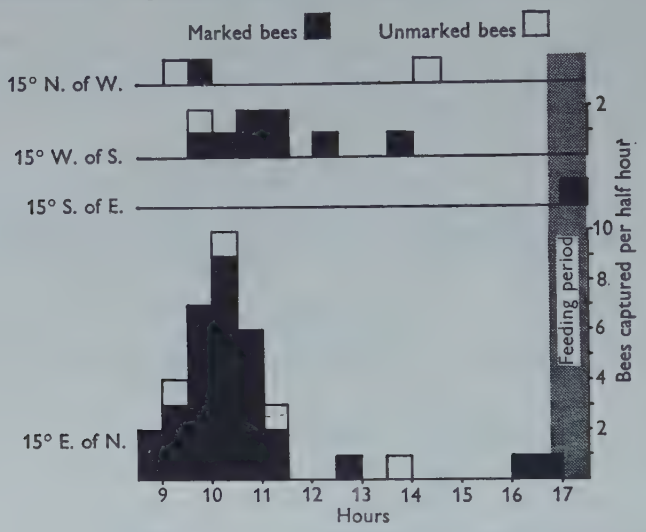

Fig. 1. Recaptures in half-hour intervals of Brazilian black bees.

The proportions of successfully orienting bees (Table 2) show remarkable agreement with the results obtained by v. Frisch (1952) in Bavaria. Thus the local honey bees of southern Brazil show the same degree of efficiency in sun navigation as the bees in Bavaria.

B. *Locally reared workers of Italian Stock from queens bred and inseminated in California*

In the evening of 11 June hive NA containing an 'Italian' queen bred and inseminated by an 'Italian' drone in California and her locally reared worker offspring, was blocked and transferred from the apiary to an experimental field of the Genetical Department some 2 km. away. On the morning of 12 June, which was a sunny day throughout, the hive entrance (facing due north) was unblocked. From 14.00 hr. onwards three stands with scented syrup dishes were moved from near the entrance in a generally western direction until at 14.36 hr. a distance of 160 m. was reached in a direction 15° N. of W. Two dishes were now removed. The small number of bees which had visited the dishes during the 'pulling out period' was now rapidly increased by offering more concentrated syrup, and until 17.30 hr. 400 bees were marked while feeding. The next morning (13 June) was a rainy one and no bees went foraging. The weather, however, improved in the afternoon, and many bees went to the syrup dish 160 m. distant and 15° N. of W., where they were fed between 15 and 17 hr. More than 100 bees marked the previous day received an

Table 1. *Visits during hourly period of Black Brazilian honey-bee workers trained in a particular direction*

Position of dish	Description of colony	Hour ending at									
		8.00	9.00	10.00	11.00	12.00	13.00	14.00	15.00	16.00	17.00
In the direction of training	LA	—	5	16	8	—	1	—	—	1	1
	LB	—	7	4	5	2	1	3	—	2	—
	LC	—	—	—	—	3	4	1	6	7	1
	LD	—	4	4	5	—	3	1	4	6	4
	Total	—	16	24	18	5	9	5	10	16	5
90° counter-clock-wise	LA	—	—	1	—	—	—	—	—	—	—
	LB	—	—	1	1	—	—	—	—	—	—
	LC	—	—	—	—	—	—	—	—	—	—
	LD	—	—	2	2	1	1	1	—	—	—
	Total	—	—	4	4	1	1	1	—	—	—
180° counter-clock-wise	LA	—	—	2	4	1	—	1	—	—	—
	LB	—	1	1	—	—	—	1	1	1	—
	LC	—	—	—	—	3	3	—	—	—	—
	LD	—	—	1	—	—	—	—	—	—	—
	Total	—	1	4	4	4	3	2	1	1	—
270° counter-clock-wise	LA	—	—	—	—	—	—	—	—	—	1
	LB	—	—	1	—	—	—	—	—	—	—
	LC	—	—	—	—	—	—	—	—	—	—
	LD	1	2	3	1	1	—	1	—	—	—
	Total	1	2	4	1	1	—	1	—	—	1

Table 2. *Comparison of v. Frisch's results with the present ones*

	Bees captured at correct dish	Bees captured on all four dishes	Remarks
v. Frisch (1949)†	20	27	Training during whole day, recapture during following morning
v. Frisch (1950)†	12	18	Training during whole day, recapture during following morning
v. Frisch (1951*a*)†	25	31	Training during whole day, recapture during following morning
v. Frisch (1951*b*)†	5	10	Evening training, morning recapture
v. Frisch (1952)	15	19	Evening training, morning recapture
Total	77=73 %	105	
Present paper:			
Colony LA	29 (32)*	36 (42)	Evening training, recapture till 12.00 hr.
Colony LB	16 (24)	21 (32)	Evening training, recapture till 12.00 hr.
Colony LC	1 (22)	2 (28)	Evening training, recapture till 12.00 hr.
Colony LD	13 (31)	26 (48)	Evening training, recapture till 12.00 hr.
Total	59=69 % (109)	85 (150)	

* ( ) recapture during whole day.
† Refers to year of experiment. Figures published 1952.

additional mark and thirty were newly marked. After sunset the hive was blocked, and transported to a field of young sugar cane some 17 km. away.

During the next day (14 June) slight cloud somewhat obscured the sun during short periods in the morning; later it was sunny throughout. Four observation stands were erected 160 m. away in the following directions: 15° N. of W., 15° E. of N., 15° S. of E., 15° W. of S. The hive was unblocked at 8.21 hr.

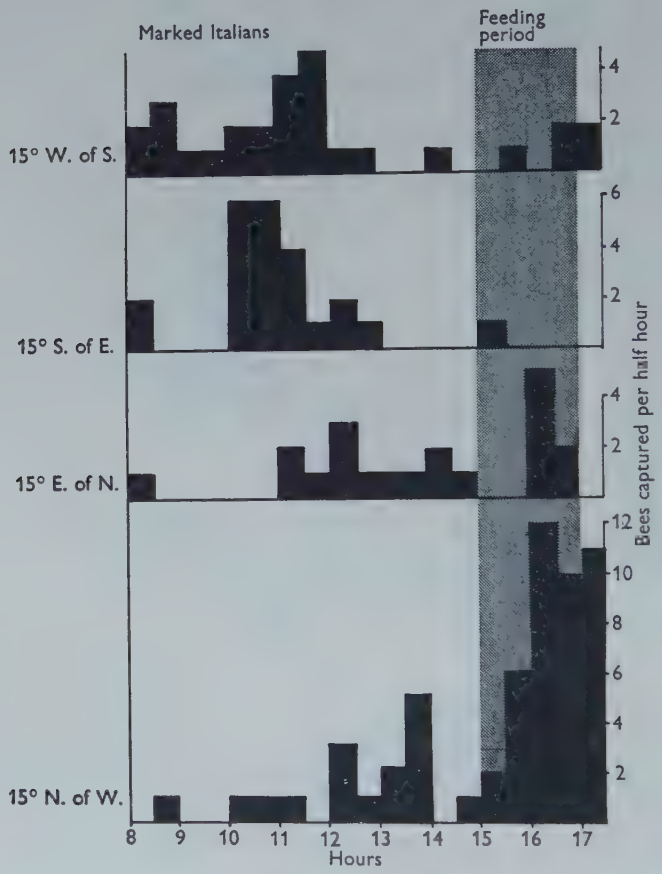

Fig. 2. Recaptures in half-hour intervals of workers from recently imported Italian queens.

Fig. 2 shows the numbers of bees captured at the four dishes, and in Table 3 these results and those of two similar experiments are summarized. The results reveal a pattern of visits quite different from that of the black Brazilian bees. Only seventy-four (40%) out of 185 captured workers were collected at the dish in the direction of training, and most of these (49) only after 15.00 hr. The visits of the bees showed a definite trend, the maximum of visitors moving counter-clockwise from dish to dish in the course of the day.

Table 3. *Recaptures during hourly intervals of marked workers from an 'Italian' queen, which after insemination had been imported from California*

Position of dish	Description of colony	Hour ending at									
		8.00	9.00	10.00	11.00	12.00	13.00	14.00	15.00	16.00	17.00
In the direction of training	NA	—	1	1	2	3	3	5	3	18	21
	NB	—	—	—	—	1	—	1	—	1	4
	NC	—	1	—	1	—	—	1	2	3	2
	Total	—	2	1	3	4	3	7	5	22	27
90° counter-clockwise from direction of training	NA	2	4	3	6	6	1	1	—	1	4
	NB	—	1	4	1	—	2	—	—	—	—
	NC	—	1	2	2	—	—	—	—	1	—
	Total	2	6	9	9	6	3	1	—	2	4
180° counter-clockwise from the direction of training	NA	2	2	6	10	3	1	—	1	—	—
	NB	—	—	1	1	1	—	—	—	1	—
	NC	—	—	1	—	1	2	3	—	—	1
	Total	2	2	8	11	5	3	3	1	1	1
270° counter-clockwise from the direction of training	NA	1	—	—	2	4	2	3	1	5	2
	NB	—	—	1	—	1	1	1	1	1	1
	NC	—	1	—	1	—	—	—	1	2	1
	Total	1	1	1	3	5	3	4	3	8	4

C. *Hybrids*

Fig. 3 shows the outcome of an experiment with colony LB of black local bees and a colony of multicoloured hybrids (HyA) descended from light 'Italian' stock (imported about 8 years previously from California) which had mated with local black drones. On the evening of 28 June colony LB was put in the middle of a large field of young sugar cane, with the hive entrance facing due east. On 29 June a number of the dark workers were trained to forage at 150 m. due south, and while feeding there between 16.00 and 17.43 hr. forty of them were marked. In another field some 5 km. away hybrids from colony HyA (hive entrance facing east) were also trained out 150 m. due south, and fifty-three of these workers were marked while feeding between 16.05 hr. and sunset at about 17.52 hr. In the evening both hives were blocked and placed side by side in a third field, both entrances facing north. The field was 4 km. from the first site and $5\frac{1}{2}$ km. from the second one. On the morning of 30 June, four observation dishes were placed at 150 m. distance in the four directions of the compass and the hives were unblocked at 07.20 hr. It was rather windy and the sun was frequently hidden by clouds. Fifty marked

hybrids but only seven marked bees from LB were caught during this day; it appears that while the few local bees were searching in the direction of training during the morning, the hybrids were not, but behaved rather like recently imported 'northerners'. Table 4 summarizes these results as far as the hybrids are concerned and the results of a second similar experiment.

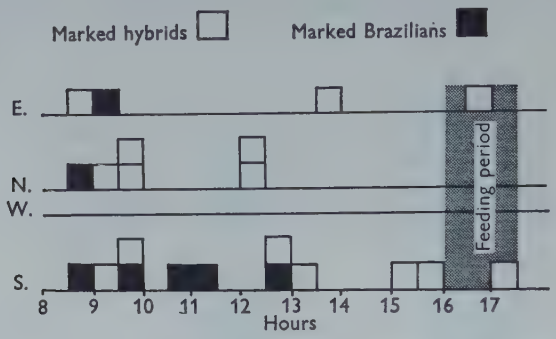

Fig. 3. Recaptures in half-hour intervals of hybrid and Brazilian bees.

Table 4. *Recapture during hourly intervals of marked hybrid workers*

Position of dish	Description of colony	Hour ending									
		8.00	9.00	10.00	11.00	12.00	13.00	14.00	15.00	16.00	17.00
Direction of training	Hy A	—	—	2	—	—	1	1	—	2	1
	Hy B	1	—	—	—	1	—	1	—	3	1
	Total	1	—	2	—	1	1	2	—	5	2
90° counter-clock-wise	Hy A	—	1	—	—	—	—	1	—	—	1
	Hy B	—	1	—	—	1	—	—	1	—	—
	Total	—	2	—	—	1	—	1	1	—	1
180° counter-clock-wise	Hy A	—	—	3	—	—	2	—	—	—	—
	Hy B	1	3	1	1	—	—	1	—	—	—
	Total	1	3	4	1	—	2	1	—	—	—
270° counter-clock-wise	Hy A	—	—	—	—	—	—	—	—	—	—
	Hy B	1	1	—	—	—	—	—	—	—	1
	Total	1	1	—	—	—	—	—	—	—	1

D. *Experiment with eight observers*

An attempt was made to test simultaneously in one experiment local honey bees, bees from a queen recently imported from the northern hemisphere, and hybrids, using at the same time eight observers instead of four. Although this experiment met only with limited success its results are given below (Fig. 4).

On 21 June colony HyC, with workers mainly descended from Italian Stock but with some black Brazilian drones in their ancestry, was put in the genetics grounds and opened facing west. On the same day hive ND, containing an Italian queen imported after insemination from California with her offspring, and hive LE,

containing black local bees, were put side by side in a field of young sugar cane some 14 km. from the site with the hybrids. Both these hives were also facing west. In the evening of 22 June bees from all three hives were gradually trained due west and between 16.00 hr. and sunset ninety-eight black Brazilians, twenty-five light 'Italians' and 273 hybrids were marked with different paints, while feeding at dishes 200 m. away from their hives in their respective fields. The three colonies were blocked in the evening and transported to an orchard 8 km. from the genetics ground and 6 km. from the sugar-cane field. Here they were put side by side all facing due north. Unfortunately, the hybrid colony was imperfectly blocked so that many marked bees escaped during the transport.

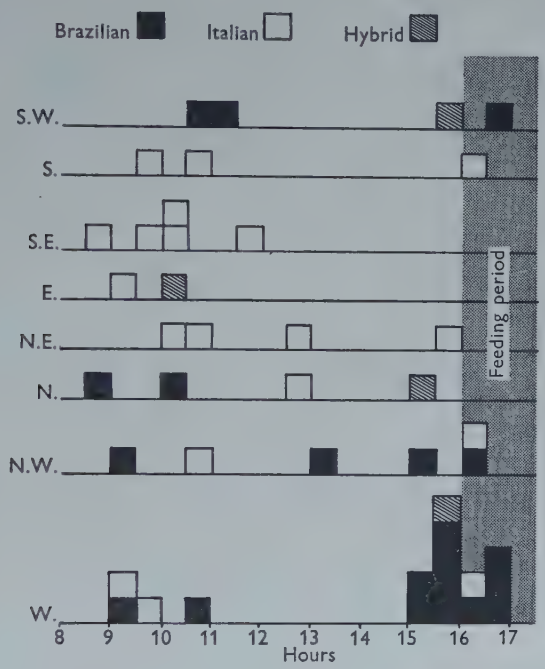

Fig. 4. Recapture in half-hour intervals of Brazilian, hybrid and Italian bees. Eight dishes.

On 23 June eight dishes were laid out in the directions indicated in Fig. 5 and the hives were unblocked at 8.26 hr. The day was mostly sunny, but a cold south wind kept the activity of the bees at a low level. The results represented in Fig. 4 may be regarded as additional evidence for the conclusions reached above concerning the orientational behaviour of the three kinds of honey bees.

DISCUSSION

It now remains (1) to correlate the visits of the foragers with the daily movements of the sun, (2) to discuss the differences of navigational behaviour of the various strains and (3) to give some indication of the way in which such differences may arise. Some results obtained by other workers are also included in the discussion.

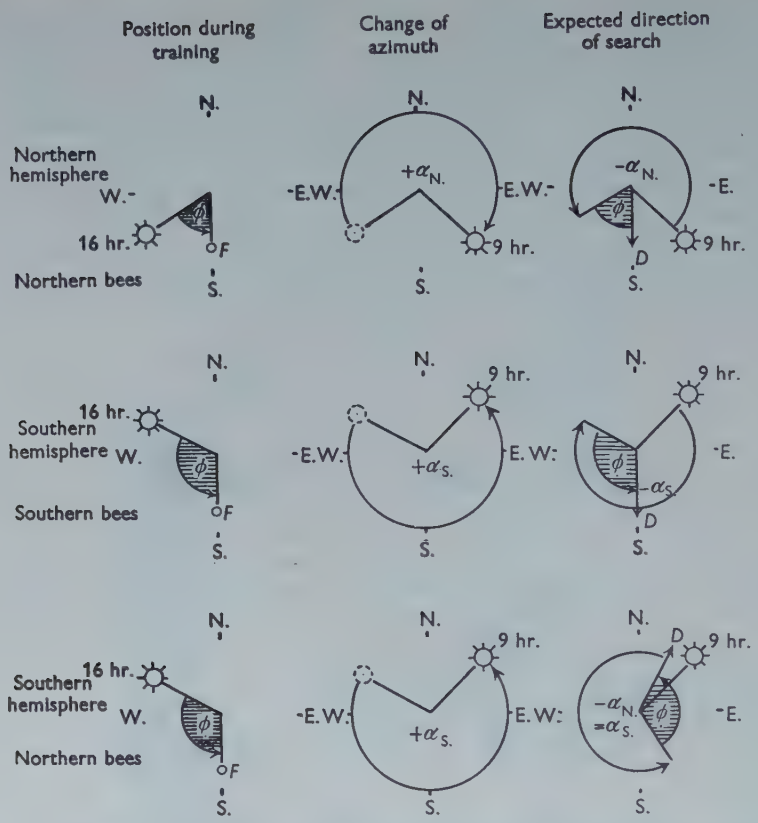

Fig. 5. Working of the reference system which enables bees to take the daily movements of the sun into account.

Our basic assumption is that the navigational abilities of bees are regulated by phenomena associated with the daily movements of the sun and in particular by the sun's azimuth. The rate of displacement of the sun's azimuth varies with locality and time. When the vertical equatorial sun moves through the zenith the azimuth changes instantaneously from east to west.* In temperate regions where v. Frisch's original observations were made, as well as in California and in Piracicaba, during the months of our observations displacement of the sun's azimuth is gradual but not quite uniform. Calculations made to correlate the visits of the bees with the exact position of the sun during the day were inconclusive and are not reported here; probably results from this type of experiment are inherently too inaccurate for such a purpose (v. Frisch, 1954). For the following interpretation a gross uniform hourly displacement of 15° per hour is being assumed.

The top row of Fig. 5 illustrates the sun navigation of bees bred and trained in temperate northern regions; fed at 16.00 hr. due south, i.e. $\phi = 60°$ counter-clock-

* A corresponding sudden change in the orientation of *Apis indica*, a close relative of the honey bee, has been reported by Lindauer (v. Frisch, 1955), who transported a colony containing direction-trained foragers across the equator and observed them afterwards.

wise, from the sun they searched due south at 9.00 hr. the following morning. In the interval the sun's azimuth has been displaced in a clockwise direction by the angle $+\alpha_{N.} = 205°$, and the hypothesis is that an internal reference system has compensated for this; this is indicated by the introduction of the counter-clockwise angle $-\alpha_{N.}$ of equal magnitude.

The behaviour in similar circumstances of southern hemisphere bees is illustrated in the middle row of Fig. 5. Here the sun moves counter-clockwise and the azimuth displacement of $+\alpha_{S.}$ is added to $\phi = 120°$. This has been compensated by the clockwise angle $-\alpha_{S.}$ and the bees thus strike out in the correct direction, i.e. due south.

The difference between the counter-clockwise compensation of northern bees in the northern hemisphere and the clockwise compensation of southern bees in the southern hemisphere could be explained in two different ways: (1) by assuming that an individual forager learns the direction of the daily course of the sun and compensates for it accordingly and (2) by assuming that the sense of compensation is innate and that an evolutionary change has occurred since the importation of the ancestors of the southern bees from their original northern home.

The results obtained with recently imported bees make the latter assumption more reasonable (Fig. 5, bottom row). Here the direction of training, as well as the position of the sun during training and observation are the same as in the experiment described in the middle row. However, instead of compensating for the counter-clockwise displacement of the sun's azimuth by the addition of the clockwise angle $-\alpha_{S.}$, the disorientated bees add the counter-clockwise angle $-\alpha_{N.}$ as if they, like their immediate ancestors, were living in the northern hemisphere. Thus they strike out in the direction *D* which is almost opposite to south, the direction of their training.* Generalizing from the assumption illustrated in Fig. 5 one can predict the direction of search of the wrongly orientated bees at any particular hour; this must change by 720° in a counter-clockwise direction in 24 hr. The bees are then expected to start in the morning in a direction about 90° clockwise from south, the direction of their training; and the direction of their search would change by about 30° per hour in a counter-clockwise direction until they reach south at 16.00 hr., the time of their previous training. The trends in visiting shown in Fig. 2 and Table 3 agree with this hypothesis.

It now remains to discuss the change in the innate compensating mechanism which we infer as having occurred during the period since the first honey bees were shipped from Portugal to Brazil, i.e. since 1530 (Schenk, 1938; Emelen, 1945). The exact time and rate of this change is unknown, but our observations on hybrids suggest that it may not have occurred very quickly. A few words may be said on one aspect of such an evolutionary transformation. Local bee keepers agree that recently imported Italian–Californian bees are at least as successful as foragers as

* A type of false orientation similar to that of the Italian–Californian bees described in the present paper seems to have occurred in individuals of the amphipod *Talitrus saltator*, which Papi (1955) transported from Italy to the Argentine. This seems to be the only experiment in the literature which has a bearing on our results.

the old Brazilians, and one might infer from that that sun navigation does not play an important role in the foraging of workers. It may, however, be important in other situations, e.g. when the bees swarm (Lindauer, 1951). While nothing is known concerning the sun navigation of queens and drones it is not unreasonable to assume that similar orientational faculties exist in the three castes of honey bees, and if so, sun navigation may be important for the nuptial flight. Thus it is possible that the change in navigational behaviour, which we infer occurred in Brazil, was brought about by the selection of the sexual forms. But no quantitative data exist on this point.

Honey bees, and especially queens, are being shipped in increasing numbers across the equator, and it might be interesting to repeat the above experiments and to see whether similar changes in navigational behaviour can be detected in different circumstances. If meliponids and trigonids should be found capable of sun navigation their transfer to the opposite hemisphere would also be of experimental interest.

SUMMARY

The sun navigation of honey bees has been investigated in a region of the southern hemisphere where the sun was moving counter-clockwise during the observations.

Foragers from a strain long established in the region were fed in the evening on a dish in a particular geographical direction and transferred overnight to a new locality unknown to them. During the next day the majority of bees were at all hours searching in the direction of their previous training.

Foragers which were the offspring of queens recently imported in an inseminated state from the northern hemisphere showed after similar training systematically false orientation on the day of observation. The direction of their search shifted by about 30° per hour counter-clockwise from a direction about 90° clockwise from the training direction in the morning to the correct direction in the evening.

Bees of hybrid (local and imported) origin also showed false orientation.

The existence of innate mechanisms is postulated compensating in northern bees for the sun's clockwise movements and in southern bees for the counter-clockwise movement of the sun. The change in the direction of compensation must have occurred during the last 425 years, and a possible mode of this evolutionary process is discussed.

The experiments described in this paper were performed during a stay in Brazil made possible by the award of the André Dreyfus prize for genetics for 1954, and the receipt of a travelling grant from the Rockefeller Foundation. Personnel and facilities of the Genetics Department of the Escola Superior di Agronomia Luiz Queiros in Piracicaba, Estado São Paulo, were kindly put at my disposal by Prof. F. G. Brieger. Dr Warwick E. Kerr, of the same department, and Dr Erico Amaral, of the Bee-keeping Department, as well as several technical assistants were indispensable helpers.

Dr R. H. Garstang of the Department of Astronomy, University College London, has advised me on the calculations of the azimuths.

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STORAGE AND UTILIZATION OF RESERVES BY THE GARDEN CHAFER, *PHYLLOPERTHA HORTICOLA* L.

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INTRODUCTION

The garden chafer is a lamellicorn with an annual life cycle. The egg, larva and pupa occur in light, well-drained soils, usually under grass. The adult lives in and above the sward and on bracken, hedges or trees nearby. The flight season lasts 4–6 weeks in May–June. Eggs are laid about $1\frac{1}{2}$ in. deep and hatch in July. From August to November or December the larvae feed on plant roots and moult twice. The fully fed third-instar larvae empty the gut and hibernate in the soil over the winter, pupating about the end of April. Adults emerge from the soil about the end of May or beginning of June, mate, lay eggs and die.

There are two main phases in this life cycle. The short larval feeding period is devoted to growth and the storage of reserves. During the rest of the year these reserves are used for living, for metamorphosis and for reproduction. This paper presents a picture of the two phases in terms of the amounts of fat, nitrogen and glycogen in the body of the individual chafer.

METHODS

All the material for analysis came from one infested field in the Lake District. Not all the samples were taken from the same generation of chafers. Most of the samples of adults were taken in the 1951 flight season, and most of the samples of larvae in the autumn of 1951 (i.e. the following generation).

All the pupae and some of the adults analysed were collected as hibernating third-instar larvae and kept in a damp atmosphere until development reached the appropriate stage. Some adults were collected from the field during the flight season. The first group of larvae to be analysed was reared from eggs under grass in flowerpots out of doors. All the larvae were of the same age. Later groups were collected from the field at intervals during the feeding period. The age given for each group is a mean value, the number of days between the mean hatching date for the year in question and the date of collection. Eggs hatched over a period of 17–28 days (mean 22 days) in the six years 1948–52 (see Milne, 1956*a*), so the age variation among the larvae in a field sample is about $\pm$ 10 days.

The individuals in each sample were weighed and assembled into groups by the

* Agricultural Research Council, Unit of Insect Physiology.

following procedure. The individuals were arranged in order of ascending weight and marked off in lots containing as many individuals as there would be groups. The individuals in each lot were then assigned, at random, one to each group. For example, if four groups were required, the four lightest individuals were allotted at random to the four groups; this allotment was repeated, always with the four lightest individuals of those remaining, until the whole sample had been divided into four groups with similar means and ranges of weight. The groups were analysed for fat, nitrogen or glycogen (usually one each for fat and nitrogen and two for glycogen). In 1951, in all but the two youngest larval samples, individuals were analysed separately. In previous years the groups were analysed as a whole.

A modified Soxhlet process was used for individual fat analysis. The chafers were dried at 90° C. for 24 hr. and stored at −3° C. Before extraction they were crushed and put into small extraction thimbles (10 × 20 mm.). The thimbles were plugged with cotton-wool and vacuum-dried to constant weight over activated alumina at room temperature. After 8 hr. extraction with petroleum ether the thimbles were again vacuum-dried to constant weight. The weight of ether-soluble material is given by subtraction and not, as in the normal Soxhlet method, by direct weighing of the extract. The direct method was used for the estimation of fat in whole groups of individuals.

Total nitrogen was estimated by the Kjeldahl method using a sodium sulphate–copper sulphate–selenium catalyst for the digestion and an ammonia microstill (Markham, 1943).

Glycogen, in groups of individuals, was extracted by Pflüger's method (Cole, 1926), hydrolysed with hydrochloric acid and the glucose estimated by Bertrand's method (Plimmer, 1920). For the analysis of single individuals a modified Pflüger's method was used (Good, Kramer & Somogyi, 1933) and the glucose obtained by hydrolysis estimated by the Hagedorn and Jensen method as described by Hawk, Oser & Summerson (1947, p. 861).

A group of newly emerged male adults was used to test the glycogen method for individuals. Seventy-two males were divided into three groups of twenty-four. One group was analysed immediately and one after the beetles had been dried at 90° C. for 24 hr. and stored at −3° C. for 2 months. The third group was also dried and kept for 2 months; it was then analysed for fat and the extracted residues analysed for glycogen. The amounts of glycogen in the three groups were 2·13, 1·65 and 1·68 mg. per beetle respectively (means). The highest value (given by the analysis of fresh material) is significantly greater than the other two ($P = 0{\cdot}001$). There is no significant difference between the latter ($P = 0{\cdot}8$).

Another test indicated that it may be safe to estimate glycogen on material pickled in Carnoy's fluid. Two groups of twelve and twenty-two feeding third-instar larvae were analysed, the former group when fresh, the latter after 24 hr. in Carnoy's fluid. The mean weights of glycogen per larva were 4·32 mg. (fresh group) and 3·76 mg. (pickled group). There is no significant difference between these two means ($P = 0{\cdot}4$). Thus there is no evidence to show that 24 hr. in Carnoy's fluid affects the glycogen content of the larva.

Two further tests indicated that fat extraction made no significant difference to the nitrogen figures. Two lots of six newly emerged male adults were analysed for total nitrogen. One lot was analysed immediately after killing and the other lot after drying and fat extraction. The mean weights of nitrogen per beetle were 2·00 and 1·92 mg. respectively. This difference was not significant ($P=0·6$). Five groups of individuals were used for the other test: 40 male pupae, 40 female pupae and three groups of female adults (40, 60 and 220 beetles respectively). Each group was halved and the halves analysed for nitrogen only and for fat and nitrogen. The individuals were not analysed separately. The nitrogen figures for each half group were expressed as nitrogen per individual and the nitrogen figures for the 'fat and nitrogen' half groups subtracted from their respective 'nitrogen only' values. The five differences were then subjected to Fisher's 'Unique Sample' test. This showed that there was no significant difference between the two methods in this instance ($P=0·3$).

STORAGE OF RESERVES

In the population sampled, newly hatched larvae weighed about 3 mg. before they had begun to feed. Their subsequent increase in live weight with age is shown by the sample figures in Table 1. Larvae weighed about 20 mg. at the first moult and 70 mg. at the second; after reaching a maximum of about 200 mg. in the third instar, the larvae weighed about 140 mg. just after going into hibernation. This decrease in weight is due chiefly to the emptying of the gut.

The water content falls during the feeding period. Water content of second-instar larvae is in the region of 90%; the two samples of second-instar larvae gave mean values of 88·0% (25-day larvae) and 87·9% (34-day larvae). The samples of third-instar larvae gave mean values of 84·2, 80·2, 80·8 and 79·1% (mean ages: 72, 94, 109 and 128 days respectively—the last sample composed of hibernating larvae). The water content of individual third-instar larvae varies little. Ranges for the four samples are: 80·9–88·0, 77·4–85·0, 80·3–81·5 and 72·7–82·7 respectively.

In the early second-instar larvae (25 days old) there was apparently little more nitrogen than in the egg (see Table 2). This result is open to criticism. There was a shortage of larvae of this age and only twenty-two were available. The larvae were dried, extracted with petroleum ether and the extracted residues analysed for nitrogen. The tests described above indicated that the double analysis could be safely carried out on pupae and adults, but this safety cannot be assumed for the young larvae where the level of nitrogen is so much lower.

By the end of the second instar (34-day sample) the level of nitrogen has risen to an average of 0·81 mg. per larva and by the middle of the feeding third instar (72-day sample) to 2·37 mg. There is little further increase and the hibernating larva contains 2·41 mg. of nitrogen (128-day sample). Nitrogen as a percentage of live weight increases steadily in the samples analysed. The five samples between 34 and 128 days give mean percentages of 1·21, 1·17, 1·49, 1·57 and 1·75 respectively. Variation of individual percentages within the samples is small (coefficients of variation, 5–7%).

Table 1. *Analysis results for larvae and pupae*

Instar	Date of collection	Estimated age in days	Live weight			Dry weight			Fat			Nitrogen			Glycogen		
			N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.
Second	—	25*	22	21·1	0·979	22	2·56	—	22	0·11	—	22	0·046	—	—	—	—
Second	24. viii. 51	34	50	69·1	1·597	20	8·38	—	20	0·85	—	6	0·808	0·049	24	0·542	0·026
Third	1. x. 51	72	44	201·0	5·275	11	32·06	2·676	11	3·84	0·552	11	2·372	0·131	22	3·455	0·418
Third	23. x. 51	94	58	204·2	4·416	12	41·32	3·029	12	5·70	0·658	12	3·005	0·115	34	3·959	0·340
Third	7. xi. 51	109	12	166·9	6·150	3	33·53	3·791	3	5·17	0·612	3	2·538	0·013	6	2·81	0·872
Third	26. xi. 51	128	117	140·1	2·601	32	29·30	1·376	32	5·01	0·332	32	2·411	0·094	53	3·96	0·216
(All hibernating)																	
Third	28. x. 48	104	59	177·1	—	59	37·29	—	59	6·67	—	—	—	—	—	—	—
(22 % hibernating)																	
Third	20. x. 49	105	205	201·8	—	58	42·10	—	58	7·83	—	89	3·461	—	58	4·38	—
(36 % hibernating)																	
Third	18. xi. 49	125	479	180·0	—	120	38·96	—	120	7·37	—	119	3·200	—	240	4·10	—
(all hibernating)																	
Male pupae	May 1950	—	40	141·2	—	40	26·13	—	20	4·08	—	40	2·500	—	—	—	—
Female pupae	May 1950	—	40	176·2	—	40	36·86	—	20	6·47	—	40	3·490	—	—	—	—

Where the standard error (S.E.) is given, the individuals were analysed separately. All means in milligrams.
* These larvae were reared from eggs.

Table 2. *Analysis results for male and female adults*

	Live weight			Dry weight			Fat			Nitrogen			Glycogen		
	N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.	N	$\bar{X}$	S.E.
Newly emerged females	46	90·1	1·835	14	27·41	1·423	14	5·39	0·516	10	2·89	0·116	22	2·48	0·100
Mature females	22	54·4	2·209	5	11·24	0·894	5	0·42	0·087	4	1·43	0·089	13	0·56	0·117
Eggs	140	1·14	—	140	0·57	—	140	0·15	—	122	0·05	—	143	0·04	—
Grass females	48	49·4	1·111	10	14·66	0·774	10	0·68	0·082	14	1·82	0·056	24	0·34	0·063
Bracken females	20	55·4	3·368	8	14·28	0·926	8	0·56	0·122	6	1·75	0·123	12	0·64	0·075
Newly emerged males	73	69·7	0·826	46	18·66	0·286	46	2·79	0·079	12	1·96	0·058	21	2·13	0·115
Bracken males	27	41·6	1·651	—	—	—	—	—	—	6	1·60	0·078	21	0·38	0·036

There is very little fat in the young larva. The amount in the body does not really begin to rise until the beginning of the third instar. From 0·85 mg. per larva at the end of the second instar, the fat content rises to 3·8 mg. at 72 days and 5·0 mg. in the hibernating larva. Variation in individual fat content among the larvae of one sample is much greater than variation in live weight, water or nitrogen. Coefficients of variation lie between 38 and 48% in the samples analysed individually for fat. Coefficients of variation for live weight, water and nitrogen lie between 15 and 25%.

There is little glycogen in the 34-day larva. By 72 days (the middle of the feeding third instar) the level has risen to 3·5 mg. This level is maintained through the rest of the third instar. Individual glycogen content figures vary widely, giving coefficients of variation of 40–75% for the third-instar samples.

From live-weight figures and from observation of the growing larva it has been suggested (Laughlin, 1956) that there are two ends to be attained by the feeding larva—growth in body size and deposition of reserve materials—and that the emphasis shifts from the former process to the latter as the feeding period progresses. Body growth takes place in the first, second and beginning of the third instars and the development of the fat body mainly in the third. The analysis data agree with this idea (see Fig. 1). Broadly speaking the nitrogen level reflects the amount of living tissue and hence the body size. The levels for fat and glycogen reflect the growth of the fat body and the amount of reserve material in the body. Nitrogen increases throughout the feeding period, while fat and glycogen are laid down mainly in the third instar (see also Chin, 1950).

THE HIBERNATING LARVA AND THE PUPA

The feeding period results in an average larva of 140 mg. (in this instance) which contains 111 mg. of water, 2·4 mg. of nitrogen, 5 mg. of fat and 4 mg. of glycogen. This material must last through the winter, provide energy and materials for metamorphosis and for the production of eggs by the female and spermatophores by the male.

Figures for three other samples of hibernating larvae and for one sample of pupae are available and are shown in Table 1. The samples were taken from the same field as the rest but were collected in different years. The analysis figures confirm the general picture given by the 1951 results and emphasize the fact that there is a considerable variation in live weight and fat content between hibernating larvae of different generations. The mean live weights of the samples cannot be compared closely because in October, November and December the mean weight of a population falls as larvae empty the gut and enter hibernation. Even after the gut is rid of all solid material a good deal of clear liquid is excreted and for the first few weeks of hibernation the weight of the hibernating larva continues to drop. However, it seems fairly certain that the samples taken in 1948 and 1951 were from populations (generations) of smaller larvae than those of 1949.

The thirty-two hibernating larvae of the 128-day sample of 1951 which were

analysed for fat show a strong correlation between live weight and fat content ($r = 0{\cdot}8215$, $P = < 0{\cdot}001$). The mean fat content of the samples also varies with the mean weight of the larvae to a certain extent. The 5 mg. of the 1951 larvae and the 7–8 mg. of the 1949 larvae indicates that, as would be expected, large larvae contain more fat than small larvae. On the other hand, the 1948 larvae were little if any bigger than the 1951 larvae and yet contained 6–7 mg. of fat. This suggests that conditions during the feeding period can affect body size and reserve stores more or less independently of each other.

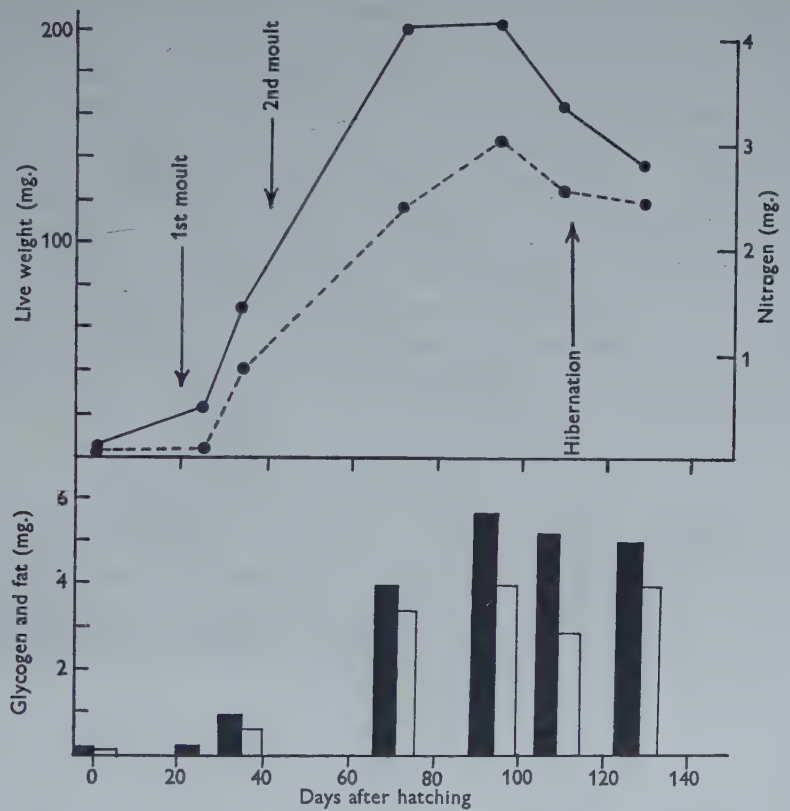

Fig. 1. The rise in live weight, nitrogen, glycogen and fat during the larval feeding period. Live weight: unbroken line. Nitrogen: broken line. Glycogen: open columns. Fat: shaded columns.

Over the winter of 1949–50, fat content dropped from nearly 8 mg. in the hibernating larva to about 5 mg. in the pupa. Nearly half the store of fat is used up over the winter. There is little fall in nitrogen content but this means no more than that little nitrogen was lost from the body.

THE EVENTS OF THE FLIGHT SEASON

The brief and generalized summary below is taken from two papers: Milne & Laughlin (1956) and Milne (1956*a*). There is much individual variation in the timing of development and of the different behaviour patterns.

In nature the pupa lies in a cell about 2 in. deep in the soil. When the adult emerges from the pupal skin, the elytra and parts of the abdominal cuticle are still unpigmented. During the next 7–8 days the cuticle hardens and darkens and the beetle moves from the cell to the surface of the sward. The rest of the beetle's life (about 14 more days) is spent partly above ground. The males run and fly over the grass and over the bracken, trees or hedges surrounding the field, resting down among the grass stems, on bracken fronds or in the trees. The females come out on to the grass, mate and return to the soil to lay their eggs. With egg laying largely completed, they too spend some time flying and resting, mostly in the bracken or trees.

At ecdysis the ovaries contain no fully developed eggs. At most one oocyte is visible as a small swelling at the base of each ovariole. The abdomen is full of fat body. Ten to fifteen days later, at 15° C., the mature female has no fat body left and the ovaries and oviducts are packed with fully developed eggs with perhaps one or two immature oocytes left in the ovarioles. In nature, egg development may be somewhat faster. Females emerging from the soil (i.e. about a week after ecdysis) have most of their eggs mature and very little fat body left (Milne, 1956*b*).

Females do not begin to lay eggs until all are mature and the fat body used up. They do not begin to feed until all or most of the eggs have been laid. The life of the adult female falls into two parts: an egg maturation and oviposition period and a period of feeding and activity after the bulk of the eggs have been laid.

UTILIZATION OF RESERVES

Two groups of female adults, collected as hibernating larvae, were analysed, the first group just after emergence from the pupa (12–36 hr.) and the second group 15–20 days later when egg development was complete. For the 15–20 days the females were kept at 15° C. in sifted soil and darkness.

Field samples of females which had laid most of their eggs were also analysed: one sample of females which had only just begun to feed and one sample off the bracken towards the end of the flight season.

The males from the collection of hibernating larvae were divided at random into five groups and killed 12–36 hr. after ecdysis. These groups were used to test combinations of analysis methods (see p. 567). A small field sample of males off the bracken at the end of the flight season was also analysed.

The collection of hibernating larvae produced 192 male and 150 female pupae. The mean weight of the female pupae was 131·4 mg. (standard error 1·724). The mean weight of forty-six newly emerged female adults was 90·1 mg. (standard error 1·835) and of twenty-two mature female adults, 15–20 days after emergence, was 54·4 mg. (standard error 2·209). Both these groups of adults were random samples of the 150 female pupae. The difference of 80 mg. between the pupa and the mature female adult is accounted for mainly in two ways: at emergence a good deal of colourless fluid is left in the cast pupal cuticle; at emergence the gut is full of accumulated excretory products which are passed out of the body in the first few days of adult life.

Analysis results are given in Table 2. All females except those in the 'newly emerged' sample were dissected before analysis. Mature eggs were dissected out and those taken from the 'mature female' sample were analysed separately. The 'grass female' sample was collected on 18 June 1951, i.e. towards the end of 'phase 1 activity' (see Milne, 1956*b*). Females were collected off the grass and dissected. Only females containing less than two mature eggs, no fat body and little or no food in the gut were analysed. Most of such females have just finished oviposition and are about to fly off to the bracken fringing the field (see Milne, 1956*b*). The 'bracken female' sample was collected on 30 June 1951, i.e. towards the end of 'phase 2 activity' and of the flight season.

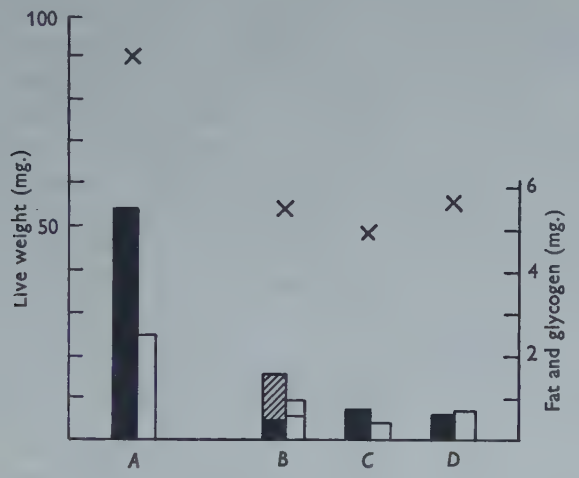

Fig 2. The utilization of fat and glycogen by the adult female. Live weight: unbroken line. Glycogen: open columns. Fat: shaded columns. *A*, newly emerged females. *B*, mature females; the upper parts of the columns show the amounts of fat and glycogen in 7·6 eggs. *C*, grass females. *D*, bracken females.

The mean live-weight figures for mature females given in Table 2 include the eggs the females were carrying. The body weight of these females (live weight minus weight of eggs) was calculated (mean 45·8 mg., standard error 1·55) and the figures put into an analysis of variance with the live weights of grass females and bracken females ($F=5{\cdot}201$, $P=0{\cdot}01$). There is no significant difference between mature females and grass females but bracken females are significantly heavier than both the former samples ($P=<0{\cdot}05$). The difference (about 7 mg.) is probably due to the large amounts of food in the guts of the bracken females.

There is no significant difference between the amounts of fat or glycogen in the last three (female) samples of Table 2. Analyses of variance gave variance ratios (F) of 1·456 ($P=0{\cdot}2$) and 2·947 ($P=0{\cdot}1$) for the fat and glycogen data respectively. The level of both substances is very low (only about half a milligram per female).

The eggs dissected from the mature female sample were analysed and the figures shown in Table 2 are the quantities per egg. This sample produced a mean number of 7·6 eggs per female. The difference in mean fat and glycogen content between

newly emerged females and the bodies of mature females is by no means accounted for by the total fat and glycogen in the eggs produced (see Fig. 2). Evidently a considerable amount of energy is used up in the metabolic processes involved in egg manufacture. In the field the female will also use energy digging in the soil. The 'mature females' were kept in loose soil, however, and will have used a minimum of energy for this purpose.

The water content of the egg is low—about 50%. The egg swells during the embryonic period, taking up about twice its own weight of water from the surroundings.

DISCUSSION

It has been shown (Raw, 1951; Milne & Laughlin, 1956) that adult feeding makes no difference to the number of eggs produced. Egg production is closely correlated with the weight of the pupa. Indeed, feeding only starts after all the eggs are mature and usually after all or most have been laid. Thus the reserves of the feeding larva form the main if not the only source of material for the production of eggs.

About half the fat and glycogen contained in the hibernating grub at the beginning of the winter is consumed before pupation. Almost all the rest is used up in the development of the adult and in maturation of the eggs. Fig. 2 shows how the still considerable fat and glycogen stores of the newly emerged adult (column *A*) are almost entirely dissipated by the time the eggs are mature (column *B*). Nor do field samples of females which have laid all or most of their eggs (columns *C* and *D*) show any recovery from this low level. Once egg development is completed, the adult female spends a very active life, burrowing in the ground to lay eggs, flying and walking about the bracken and, in some cases, taking off on long distance flights to colonize new areas (see Milne, 1956*b*). There is no store of fat and glycogen left to provide all this energy (the half milligram of fat and glycogen that is left could probably not support flight for more than about 20 min.,* even if both sources of energy were used). Thus feeding by the adult female is essential for continued activity in the second half of adult life.

SUMMARY

1. Samples of all stages of *Phyllopertha horticola* L. have been analysed for fat, total nitrogen and glycogen.

2. Total nitrogen increases throughout the larval feeding period, while fat and glycogen are laid down mainly in the latter half.

3. From November, when the third-instar larva goes into hibernation, until June, when the eggs have been matured and laid, no food is taken.

4. Of the store of fat and glycogen in the hibernating larva at the beginning of the winter, half is used up by the time the adult emerges. The other half is used in the formation of eggs.

* This figure was calculated knowing that (*a*) a bee of about 100 mg. uses about 10 mg. of sugar per hour in flight (Wigglesworth, 1950, p.392) and (*b*) the calorific value of fat is 2·2 times that of carbohydrate; and assuming that (*a*) a garden chafer adult of about 50 mg. uses about half the sugar used by a bee of 100 mg. and (*b*) both fat and glycogen are available for use in flight.

5. Adult feeding provides energy for the post-oviposition activity period.

I am very grateful to Prof. V. B. Wigglesworth and to Dr. A. Milne for many helpful discussions. I would also like to thank Mr. A. Thompson, of the Department of Agricultural Chemistry in this college, for discussions on the methods used and for the loan of apparatus and other facilities.

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STUDIES IN DIURNAL RHYTHMS

VII. HUMIDITY RESPONSES AND NOCTURNAL ACTIVITY IN WOODLICE (ISOPODA)

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INTRODUCTION

It has been suggested that there may be some relationship between the time of day at which locomotory activity takes place, and the water relations and humidity responses of the terrestrial arthropods that lack an effective waterproofing epicuticular layer of wax (Cloudsley-Thompson, 1954). Woodlice have been selected as suitable material for investigating this point because they form a reasonably homogeneous group containing several common species that show different degrees of adaptation to life on land (Edney, 1954).

MATERIAL

The species used in this investigation were *Philoscia muscorum* (Scop.), *Oniscus asellus* L., *Porcellio scaber* Latr. and *Armadillidium vulgare* (Latr.). Adult animals were nearly always used, but the sexes and colour varieties were not separated. Many of the *Ph. muscorum*, *P. scaber* and *A. vulgare* were kindly sent to me from Box Hill, Surrey, by Mr J. H. P. Sankey, the remaining material being collected from my own garden in Esher, Surrey. These species have been shown to stand in the following order as regards the rate of water loss by transpiration: *Philoscia muscorum* > *Oniscus asellus* > *Porcellio scaber* > *Armadillidium vulgare* (Edney, 1951).

HUMIDITY RESPONSES

The responses of the woodlice to humidity were tested by means of choice-chamber apparatus, using a technique already described (Cloudsley-Thompson, 1952). Experiments were carried out at room temperature ($18 \pm 2°$ C.) and in darkness, since it has already been found that in *O. asellus* the intensity of the response is affected by light. The results obtained are given in Table 1 and show that the intensity of the humidity response decreased from *Ph. muscorum* through *O. asellus* and *P. scaber* to *A. vulgare* under the conditions of the experiment. They are in agreement with those of Waloff (1941). At the same time less *A. vulgare* were stimulated into activity by drought than *P. scaber*, and less of this species than of *O. asellus*. The small number of *Ph. muscorum* recorded as 'moving' appears to be an exception to the general tendency. This species was found to be much more easily disturbed than the others however, and the slightest vibration caused the animals to dash off at a surprising speed.

The fact that the intensity of the reaction increased in the second halves of the experiments, when the animals were becoming desiccated, may perhaps be correlated with the fact that when exposed to dry atmospheres water is at first lost mainly from the outer layers of the cuticle external to a lipoid barrier, and only later from the body fluids (Bursell, 1955).

Table 1. *Percentage of woodlice moving, or stationary on the dry side* (50% R.H.), *in the middle or on the moist side* (100% R.H.) *of a choice chamber at room temperature* (18 ± 1° C.) *in darkness*

	P. muscorum	*O. asellus*	*O. asellus* (from Cloudsley-Thompson, 1952)	*P. scaber*	*A. vulgare*
			First 75 min. exposure		
Moving	4·8	12·4	9·2	8·8	1·2
On dry side	0·4	7·6	12·8	23·2	56·4
In middle	4·0	11·2	12·4	16·4	7·6
On moist side	90·8	68·8	65·6	51·6	34·8
Intensity of reaction	**38·7**	**5·6**	**3·8**	**1·9**	**0·62**
			Second 75 min. exposure		
Moving	0·0	6·0	6·8	6·4	3·2
On dry side	0·0	6·0	1·2	13·6	43·7
In middle	0·0	12·0	6·4	16·0	10·7
On moist side	100·0	76·0	85·6	64·0	42·4
Intensity of reaction	**∞**	**6·8**	**20·2**	**3·3**	**0·97**
			Mean of 150 min. exposure		
Moving	2·4	9·2	8·0	7·6	2·2
On dry side	0·2	6·8	7·0	18·4	50·0
In middle	2·0	11·6	9·4	16·2	9·2
On moist side	95·4	72·4	75·6	57·8	38·6
Intensity of reaction	**96·4**	**6·2**	**6·9**	**2·5**	**0·79**

EFFECT OF TEMPERATURE ON HUMIDITY RESPONSES

The effect of temperature on the responses of the woodlice to humidity was investigated by carrying out experiments similar to those described above, but at different temperatures, viz. 3 ± 2° C., and 30 ± 2° C., and comparing them with the results already obtained at 18 ± 2° C. These experiments were all of 75 min. duration, and the results obtained are presented in Table 2. From these it can be seen that the intensity of the humidity response was less at the lowest temperature where the figures for the four species are not significantly different from one another ($P = 0{\cdot}05$). At the highest temperature not only were a greater proportion of all species moving about, but there was a tendency for the humidity response to be decreased in *Ph. muscorum* and *O. asellus*. This can be explained by the fact that the upper lethal temperature for these species is lower than that for *P. scaber* and *A. vulgare* according to Edney (1951). Consequently there may be a tendency as the lethal temperature is approached for the animals to move into dry air where water loss by evaporation and consequent cooling takes place more rapidly, for near the upper lethal temperature heat becomes an even more important factor than humidity.

Table 2. *Effect of temperature on the response of woodlice to humidity. Percentage of woodlice moving, or stationary on the dry side* (50% R.H.), *in the middle, or on the moist side* (100% R.H.) *of a choice chamber in darkness at various temperatures. The figures for the intensity of the reaction in the four species at* $3 \pm 2°$ C. *do not differ significantly from each other* ($P = 0{\cdot}05$)

Temperature		*P. muscorum*	*O. asellus*	*P. scaber*	*A. vulgare*
$3 \pm 2°$ C.	Moving	2·5	0·7	0·9	1·2
	On dry side	22·0	21·5	30·0	23·2
	In middle	16·0	13·5	23·4	27·2
	On moist side	59·5	64·3	45·7	48·4
	Intensity of reaction	**2·2**	**2·5**	**1·4**	**1·7**
$18 \pm 2°$ C.	Moving	4·8	12·4	8·8	1·2
	On dry side	0·4	7·6	23·2	56·4
	In middle	4·0	11·2	16·4	7·6
	On moist side	90·8	68·8	51·6	34·8
	Intensity of reaction	**38·7**	**5·6**	**1·9**	**0·62**
$30 \pm 2°$ C.	Moving	16·0	24·8	14·2	24·2
	On dry side	9·6	14·4	18·6	19·8
	In middle	11·2	9·3	10·6	11·3
	On moist side	63·2	51·5	56·6	44·7
	Intensity of reaction	**4·5**	**3·0**	**2·6**	**1·9**

SENSORY PHYSIOLOGY OF HUMIDITY RESPONSES

Although saturation deficiency varies considerably with temperature, relative humidities maintained by sulphuric acid-water mixtures are relatively constant over a wide range (Solomon, 1951, etc.). The fact that, at a particular relative humidity, the intensity of the humidity response of woodlice increases with temperature suggests that these animals respond to the saturation deficiency of the atmosphere rather than to its relative humidity. On the other hand, in insects which have a discrete epicuticular layer of wax and do not lose much water by evaporation except at temperatures above the critical transitional temperature of this wax layer, it is believed that the humidity receptors function hygroscopically rather than as evaporimeters (Kennedy, 1939; Pielou & Gunn, 1940; Lees, 1943; Wigglesworth, 1953).

The sensory physiology of the terrestrial Isopoda appears to be little known. As a result of histological and morphological investigations, Abraham & Wolsky (1930*a*) claimed that a sense organ located on the inner lobe of the second maxilla was an organ of taste, and in a second paper (1930*b*) they described the peg sensilla on the antennule as an olfactory organ ('Geruchsorgan'), but no experimental evidence was provided in either case in support of these views. The apparent similarity in structure between the antennal sense organs of woodlice and the basiconic sensillae of millipedes (Diplopoda) suggests that they may in fact have a similar function—that of appreciating contact chemical stimuli. It has been shown that millipedes are attracted to 1% sucrose solutions (Cloudsley-Thompson, 1951), and it was therefore decided to test the responses of *O. asellus* to this. Groups of ten woodlice were placed in choice-chamber apparatus at room temperature in

darkness. The two halves of the floors of the arenas were damped respectively with distilled water and with 1% sucrose solution, or with 1% and with 2% sucrose solutions. The number of animals in each half of the arena was counted at hourly intervals over periods of 3 days; the woodlice were thoroughly stirred up with a glass rod between readings so that there were equal numbers in each half of the arena. At the same time the usual precautions were taken to ensure that any factors external to the choice chambers were controlled. As a result of ten sets of experiments it was found that *O. asellus* showed a preference for filter-paper damped with distilled water (59·5%) to one damped with 1% sucrose (40·5%), and this, in turn, was preferred (58·5%) to filter-paper damped with 2% sucrose solution (41·5%).

The reaction was not affected when the mouthparts were painted over with a solution of cellulose in amyl acetate, but was abolished when the terminal segments of the antennae were removed or painted over. The humidity response was not influenced by either of these operations nor by covering the antennules or pleopods with paraffin wax. It persisted even when the antennal cones, antennulae, mouthparts and pleopods were all coated with paraffin wax, although some of the animals appeared to be somewhat hampered by the weight they were carrying.

It is probable therefore that whereas the antennal cones may be contact chemoreceptors, no specific sense organs are concerned with the appreciation of humidity. As in millipedes (Cloudsley-Thompson, 1951) it is probable that an orthokinesis is engendered by a general effect of dehydration, and this would explain the fact that the response appears to be correlated with saturation deficiency and not with relative humidity as obtains in those terrestrial arthropods that possess specific humidity receptors.

RESPONSES TO LIGHT

The responses to light of the four species of woodlice were compared, again by means of choice-chamber apparatus, in which alternatives of diffuse daylight (registering approximately 3·0 units on a Weston Master II photoelectric meter) and darkness were offered. The methods used were as before (Cloudsley-Thompson, 1952) and the results obtained, in percentages of animals in the dark half of the arena, were as follows: *Ph. muscorum*, 100%; *O. asellus*, 80%; *P. scaber*, 79%; *A. vulgare*, 72%. The species therefore show a graduation in the intensity of their responses to light which parallels that of their rates of water loss by evaporation in dry air.

INTENSITY OF NOCTURNALISM

The percentages of locomotory activity taking place during the hours of darkness in the four species were compared by means of the aktograph apparatus previously used (Cloudsley-Thompson, 1952). This was placed in a large incubator in which light and heating were controlled by means of time switches. Individual animals were placed in the arena of the apparatus for periods of 5 days. During this time they were in darkness from 06.00 to 18.00 hr. G.M.T. and were illuminated with a 15 w. pearl bulb at 20 cm. distance from 18.00 to 06.00 hr. The intensity of light falling on the floor of the arena registered 75 units on a Weston Master II photoelectric meter. The temperature, recorded by means of a thermo-hygrograph, fell to

20 ± 1° C. during the hours of darkness and rose to 24 ± 1° C. while the light was on. The floor of the arena was lined with damp filter-paper so that the relative humidity was maintained at a high level and did not fluctuate with temperature. The kymograph records were analysed as block histograms; the number of times that a woodlouse crossed the axis and caused the arena to rock in each 3 hr. period during the 5-day experiments was counted.

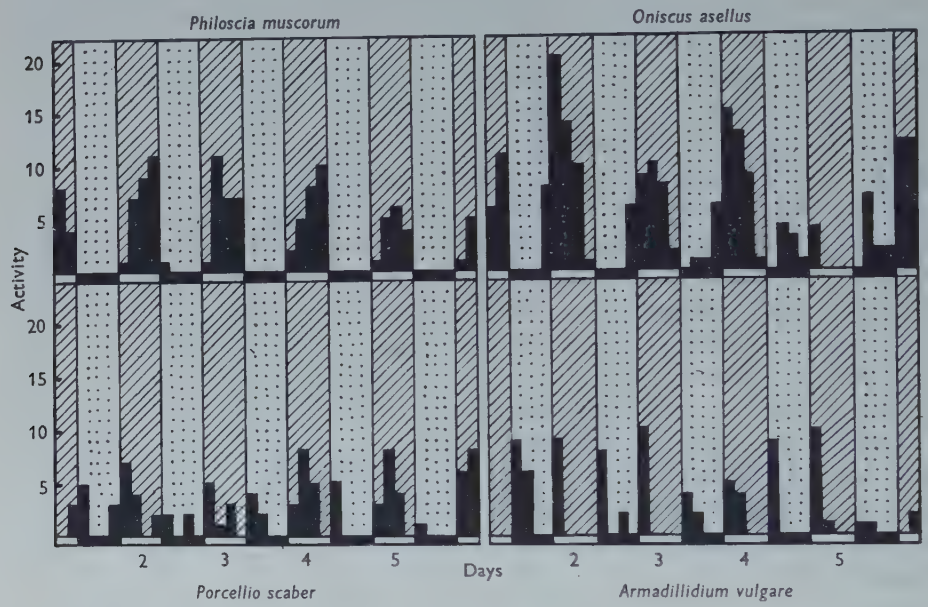

Fig. 1. Locomotory activity of woodlice in aktograph apparatus, exposed to alternating light and darkness. The light periods from 18.00 to 06.00 hr. are indicated by stippling, the dark periods from 06.00 to 18.00 hr. by hatching. Ordinate: activity, represented by the number of times the experimental animal crossed the axis of the arena. Abscissa: time in days. The black strips indicate the 12 hr. periods from 18.00 to 06.00 hr., the white strips from 06.00 to 18.00 hr. Further explanation in the text.

In preliminary experiments a curious anomaly became apparent in that *Ph. muscorum* showed practically no periodicity in alternating light and darkness, although this species is normally the most strongly photonegative (see above). Presumably, kinetic activity was stimulated by the light. However, when a refuge in the form of a thin rectangular sheet of zinc metal measuring 2 × 4 cm. bent along its longitudinal axis in the form of a V was placed in an inverted position on the floor of the arena, a well-marked 24 hr. cycle of activity and rest was recorded. Under cover of its refuge the small woodlouse was able to shelter from the light and seldom wandered into the open except during the hours of darkness. The activity cycles of the other species were far less affected by the absence of a refuge; indeed, in the case of *A. vulgare* no difference was noted in the kymograph records whether shelter was present or not. Nevertheless, a refuge was present in all the experiments analysed below. The first 6 hr. record of each animal was not counted to allow time for the disturbing influence of being placed in the arena to wear off.

The percentage of locomotory activity that took place during the periods of dark-

ness, based on at least five 5-day experiments with different individuals of each species, was found to be as follows: *Ph. muscorum*, 92%; *O. asellus*, 77%; *P. scaber*, 71%; and *A. vulgare* 60%. Thus the species with the highest rate of water loss through transpiration are the most intensely nocturnal in habit, and vice versa. Some typical histograms of kymograph records are given in Fig. 1.

At the same time the mean activity per 24 hr., represented by the average number of times that the experimental animal crossed the axis of the arena, was as follows: *Ph. muscorum*, 16; *O. asellus*, 33; *P. scaber*, 23; and *A. vulgare*, 14.

DISCUSSION

The results obtained above can be related to what is known of the physiology and ecology of the species investigated. All woodlice require saturated air or a moist substrate in their permanent habitats but differ as regards tolerance of sub-optimal conditions during their wanderings (Edney, 1954). Thus *Ph. muscorum*, which has the highest rate of water loss by transpiration, is the most markedly nocturnal and photonegative, and shows the most intense reaction, whereas the reverse is true of *A. vulgare*, the species with the highest upper lethal temperature and the lowest transpiration rate. Of all woodlice the species best adapted to terrestrial life is perhaps *Hemilepistus reaumuri* Aud. This occurs in desert regions and is active mostly during the hours of daylight preceding dusk and after dawn (Cloudsley-Thompson, 1956), but even here the permanent retreats of the animals are deep, moist burrows.

The differences between the figures for the intensity of the humidity and light responses of *O. asellus* in the present work and those obtained previously (Cloudsley-Thompson, 1952) may perhaps be due to seasonal changes in the physiology of the species, for as Perttunen (1953) has shown in the case of the millipede *Schizophyllum sabulosum* (L.) a marked seasonal change in the initial humidity reaction occurs in undesiccated specimens: the dry, summer reaction is gradually reversed to moist in the autumn.

Although as Bursell (1955) has shown, the transpiration rates of woodlice are very small compared with the loss from a free surface of water, nevertheless they are on the whole much greater than those from arthropods possessing an epicuticular layer of wax. There are two obvious ways in which small animals can escape becoming desiccated on dry land. One is to avoid dry places and remain most if not all the time in a humid environment as a result of physiological orienting mechanisms; the other to acquire a relatively impermeable integument and the physiological and morphological specializations that allow respiration, excretion and so on to take place without excessive loss of water. The writer feels no need to modify his opinion that the terrestrial Isopoda represent a group that has exploited the first of these methods (Cloudsley-Thompson, 1954).

SUMMARY

The species of woodlouse investigated are known to stand in the following order as regards the rate of water loss by transpiration: *Philoscia muscorum* > *Oniscus asellus* > *Porcellio scaber* > *Armadillidium vulgare*. The intensity of the responses of the

four species to humidity at room temperature ($18 \pm 2°$ C.) are compared and are shown to stand in the same order. The intensity increases as desiccation proceeds.

At low temperatures ($3 \pm 2°$ C.) the intensities of the reactions to humidity are much reduced and are the same in all species; at higher temperatures ($30 \pm 2°$ C.) the humidity responses of *Ph. muscorum* and *O. asellus* are again somewhat reduced, and it is suggested that this may be correlated with a lower thermal death-point and the need to effect a reduction in body temperature by evaporation.

The antennal cone sensillae are shown to be contact chemo-receptors; no specific sense organs are concerned with the appreciation of humidity. The response is correlated with saturation deficiency rather than with relative humidity and is probably engendered by dehydration. *Ph. muscorum* is the most strongly photo-negative of the species and there is a graduation through *O. asellus* and *P. scaber* to *A. vulgare* which is the least so.

Ph. muscorum is also shown to be the most intensely nocturnal in habit, *A. vulgare* the least. It is therefore suggested that the degree of nocturnal activity is correlated with the ability to withstand water loss by transpiration. The results obtained are discussed in relation to the ecology of the species.

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FACTORS WHICH INFLUENCE THE ACQUISITION OF FLAGELLA BY THE AMOEBA, *NAEGLERIA GRUBERI*

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INTRODUCTION

During the course of some investigations upon the behaviour of cells isolated from sponges (*Sycon* sp.) by squeezing the sponge through fine silk, the behaviour of the normally flagellated collar cells, or choanocytes, aroused particular attention and curiosity. Especially was this so because of the apparent ease with which these cells could lose first their collar, then their flagellum, and finally become creeping amoeboid cells, only to reacquire the flagellum again, and sometimes the collar also, when conditions became favourable to this change. This phenomenon, which is interesting enough in itself, may also have implications of much wider significance.

In the early developmental stages of sponges, the larva can be more or less clearly subdivided morphologically into an anterior region of flagellated cells and a posterior region of amoeboid cells, or at least of cells which have no flagella. In some species (e.g. *Clathrina blanca*) these posterior cells may be restricted at first to a single pair of archaeocytes. In *Sycon*, on the other hand, the embryo is fairly evenly divided equatorially into the two classes of cells, though in this species the cells in the equatorial zone give the impression of being somewhat intermediate in character. These intermediate cells, though similar in shape to the anterior cells, are much more granular, and in this way resemble the cells of the posterior half.

This antero-posterior division of the embryo is not, however, a peculiarity of the sponges, for in many other groups of invertebrates beside the Porifera, the embryo or larval animal is clearly polarized into an anterior (animal) pole and a posterior (vegetal) pole. In some species, this polarization and subdivision into zones is as obvious as it is in the sponges, and the cells of the anterior pole are ciliated or flagellated; in others, the gradient from animal to vegetal pole only expresses itself in more subtle ways. Nevertheless, throughout a wide range of animal types, there is evidence for an axial gradient in some form or another. The difference in character between the anterior 'animal' cells, which primarily give rise to the external, sensory and protective layers of the organism, and the posterior, more digestive and vegetative cells which habitually enter the inner layers, is thus a very widespread and fundamental difference.

Whatever views one may hold on the origin of Metazoa from Protozoa, on the respective claims of the ciliates or flagellates as metazoan ancestors, or on the phylogenetic positions of the Porifera, Coelenterata and Turbellaria, the existence

of an antero-posterior gradient in so many organisms is a phenomenon which has been often described, but seldom explained.

Since this gradient frequently shows itself by the presence of flagellum-bearing or ciliated cells anteriorly and of more amoeboid cells posteriorly or centrally in the embryo, with often an intermediate zone of cells separating the two clear types, the nature of the difference between flagellate cells on the one hand and amoeboid cells on the other is obviously a problem worthy of investigation. More particularly does the problem become interesting when the different forms of behaviour are separated not in space, as in the two halves of the embryo as just outlined, but in time, as in isolated choanocytes or in the behaviour of certain amoebae.

Moreover, the nature of the physiological distinctions between flagellated or ciliated cells on the one hand and non-ciliated cells, which often produce 'mucin', on the other, has repercussions which may be applicable to problems of cellular differentiation even in the higher vertebrates. For example, when the perfectly uniform and normally keratinizing skin of the chick embryo is treated in tissue culture with high doses of vitamin A, the cells change both their morphological character and their physiological behaviour (Fell & Mellanby, 1953). Some of the cells acquire cilia, others secrete 'mucin'. It would be fascinating to know what are the factors operating in this change, why the change leads to a diversity of cell behaviour and how the vitamin A acts. These observations on the effects of vitamin A also raise questions as to what is the relationship between the ciliated and the non-ciliated cells in such epithelia as that of the respiratory tract, and whether there is some antithesis between the formation of flagella (or cilia) and the production of mucoprotein.

Now it has been known for many years (Schaudinn, 1896; Schardinger, 1899) that, in certain amoebae, of which *Naegleria gruberi* is an excellent example, the individual cell may exist in one of two forms; it may either live as a typical, creeping and phagocytic amoeba, or it may, under certain conditions, acquire one or more flagella and become free-swimming. This change, from the amoeboid to the flagellate form, almost invariably occurs when the amoebae which have been growing, for example, on an agar-meat-extract slope, are transferred to pure water.

An investigation of the nature and cause of this change from one form of activity to another seemed therefore likely to yield information of some interest, not only in its immediate relation to the life of these special amoebae, but also because of its possible application to the wider biological issues already surveyed, namely, the relationship between cells with flagella (or cilia) and those in which movement is essentially amoeboid; and, as a sequel to this, to one of the main characteristics which helps to define the antero-posterior gradient of so many invertebrate, and indeed vertebrate, embryos.

METHODS

The amoebae (*Naegleria gruberi*), which were originally obtained from the type collection of Protozoa, Algae, etc. in the Botany School, Cambridge (1518, 1), were kept as stock cultures on agar slopes, in test-tubes stoppered with wool and closed

with a paper seal. The composition of the agar was as follows: 7·5 g. powdered agar was dissolved in distilled water and filtered before adding 0·5 g. 'Lemco' meat extract and 1·0 g. glucose and then making up to 500 ml. with distilled water. The tubes containing about 4 ml. of medium were sterilized in an autoclave and then cooled to form slopes. When the amoebae were in need of subculture, each slope was washed with about 5 ml. of sterile distilled water, and the amoebae spun down in a centrifuge at about 3000 r.p.m. for 3–4 min. The usual precautions against infection from outside sources were observed. These amoebae were then re-suspended in another 5 ml. of sterile distilled water and re-centrifuged. The washed amoebae were then suspended in a smaller volume (0·5–1·0 ml.) of sterile distilled water, appropriate to the cell density, and single drops from this suspension of cells were then used for seeding fresh agar–Lemco slopes. For building up the stock cultures for use in experiments it has been found better not to wash the amoebae too thoroughly. A high density of amoebae can be better maintained if they are transferred to the agar slopes with their accompanying bacteria. For experimental studies, however, more thorough washing is required.

The stock cultures normally grow freely for about 10 days at room temperature (generally 15–25° C.). During the first 3 or 4 days the amoebae are large and active; many polynucleate cells are found. After that time the cells become smaller, the growth declines and the number of amoebae in the form of cysts increases rapidly. Such cysts, if suspended on fresh agar slopes, soon produce active amoebae. For experimental purposes, cultures between the ages of 3 and 7 days have given the most uniform and satisfactory results, but there is room for a more detailed investigation into the optimal age and into the effects of culture conditions on the numbers of cells which acquire flagella when treated with water. It has been noticed that sometimes nearly all the amoebae become flagellated in a very short time, not more than 2 or 3 hr., while at other times there are never more than a relatively small percentage of flagellate forms, and these may not appear till the amoebae have been in water for six or more hours. In winter, when there is a tendency for the cells to remain in the amoeboid form in spite of their transference to distilled water, it has been found beneficial to keep the stock cultures well illuminated. Other observers (Schardinger, 1899) have noted a marked tendency for the flagellate form to increase in numbers with rise of temperature up to 34° C. One of the factors in this variability in the time of appearance may thus be the fluctuation of room temperature, but there must certainly be other factors also, since a few preliminary experiments have not so far confirmed the observations of Schardinger.

The cells normally feed on bacteria and, in healthy conditions, the populations of bacteria and amoebae seem to control each other and reach a sort of steady state. In spite of this initial necessity for bacteria, the cultures have been kept under otherwise aseptic conditions, i.e. all subculturing has been done with simple aseptic precautions; glassware and culture media have been sterilized and all tubes sealed when not in use. If these elementary precautions are not taken, the cultures quickly become contaminated with moulds, yeasts, etc., and these are then difficult

to eradicate. The presence of bacteria is, of course, a major source of difficulty and of irregularity in results, and a satisfactory sterile synthetic medium would offer enormous advantages. Brent (1954) has produced a satisfactory sterile medium for *Tetramitus* by using autoclaved bacteria, and Reich (1935) has grown *Mayorella* on a peptone medium.

In order to investigate the effects of any substance on the activity of the amoebae, the substance was first dissolved in sterile distilled water and the hydrogen-ion concentration adjusted approximately to pH 7. Serial dilutions of this solution were then made in distilled water in test-tubes which had been very thoroughly cleaned, sterilized with absolute alcohol, and rinsed out with sterile distilled water. 0·5 ml. of solution were placed in each tube and one drop of a well-mixed and uniform suspension of amoebae was then added to each tube. To make this suspension the contents of two or three culture tubes (3–7 days' growth) were washed and centrifuged at least three times with distilled water to obtain them relatively free from bacteria, though by no means sterile. After the last washing the cells were taken up in 3 or 4 ml. of distilled water according to their density. Two culture tubes usually gave enough suspension to seed about sixty experimental tubes. Three may be necessary in winter. It has been found advantageous not to have too many amoebae present, otherwise the subsequent counting becomes difficult (about 2000 cells per cu.mm. is a convenient concentration for the original suspension). From time to time, a sample drop of as nearly as possible constant volume (about 2 cu.mm.) was taken with a marked Pasteur pipette from each experimental tube and these drops were placed on a microscope slide, four at a time. The number of flagellates which could then be counted in a single 'journey' of the microscope field ($\frac{2}{3}$ in. objective) round the edge of each drop were then noted, and the population in the various tubes compared in this manner. This semi-quantitative method shows quite clearly at what concentrations of the substance under investigation the amoebae assume the flagellate form; further examination of the 'creeping' amoebae shows whether the solution has any differential action on the two forms of activity. Several control tubes, containing a medium of distilled water only, were always seeded and counted at the same time, so that the behaviour of the batch of cultures as a whole could be to some extent standardized.

Estimation of pH values in the observation to be described have all been made colorimetrically with phenol-red, cresol-red, thymol-blue or B.D.H. universal indicators. Phenol-red was often incorporated in the medium without any harmful effects. The values of pH given are therefore only approximate but are considered adequate, in view of the reaction of the amoebae to this aspect of their environment. Moreover, in experiments lasting for any length of time in fluids as dilute as those used, the initial pH may differ considerably from the final pH because of the production or diffusion of CO_2 or other metabolites. In general, results obtained during the first 6 hr. or so are considered to be directly related to the medium as constituted, perhaps allowing for diffusion of CO_2, etc., but, in experiments of longer duration, subsidiary and confusing effects may be produced by bacterial growth, etc. These latter effects become particularly important in media containing

food substances like glucose. It should, therefore, be stressed that the investigations described in this paper have been aimed more at finding the broad principles involved in the change from amoeba to flagellate and less at the detailed analysis of any one set of conditions.

MORPHOLOGICAL CHANGES

The amoebae, *Naegleria gruberi*, when growing on an agar surface in the presence of bacteria may, like other amoebae, assume almost any shape: the cells move in what appears to be a more or less random manner; though this, in fact, is probably determined by local changes in the environment. The amoebae tend to spread out and to maintain spaces between themselves, but they may cluster round a rich food supply or, if a thick population is released into a drop of fluid on a cover slip, spread centrifugally in more or less serried ranks. Rounded pseudopodia may appear at any point on the surface of the cell, and one or more contractile vacuoles come and go within the cytoplasm without any definite or obviously constant location. The nucleus also moves freely about within the cell. It contains a conspicuous nucleolus, and is surrounded by numerous highly refractile granules; these give the nuclear membrane a beaded appearance and are particularly conspicuous in preparations viewed by phase contrast. When several contractile vacuoles form they usually fuse before evacuating.

When the cells are washed almost free from bacteria and allowed to settle in distilled water on the clean glass surface of a cover-slip or slide, certain characteristic changes of morphology follow (Fig. 1). After a short time, often about an hour, a tendency towards a definite polarity develops in the cells. Rounded pseudopodia are pushed out more in one direction than another and the organism naturally tends to move in that direction. This direction is not rigidly fixed, but the amoeba is fairly clearly developing an anterior and a posterior half, the former sending out rounded pseudopodia and the latter developing a more or less definite tail-zone or 'uroid' to which numerous bacteria may often be seen to be attached possibly by a mucoid secretion (Fig. 1; 4) (cf. Hollande, 1945; Martin & Lewin, 1914). The surface of this tail-zone appears to be in some ways more stable and rigid, in the sense that pseudopodia develop less readily from it and, when they do so, they are not the usual type but take the form of fine thread-like pseudopodia generally directed backwards, approximately parallel to the antero-posterior axis (Fig. 1; 5). They sometimes appear to be the result of the cell creeping forward but leaving behind points of attachment to the glass which remain connected by ever-extending threads of protoplasm.

These filiform pseudopodia were described by Pietschmann (1929) and can be seen to come and go; they may move about slowly from side to side, or may bend sharply and fold in on themselves, thickening as they do so, to be finally reabsorbed into the cell body once more. At this stage in the progressive change of form, the contractile vacuole takes up a position near the posterior pole, and, after it has collapsed, it now reappears as a group of five or six small vacuoles very often in

the form of a rosette (Fig. 1; 6). These small vacuoles progressively enlarge, fuse together and evacuate, regularly, with a period of about 30 sec. This period remains almost constant during the subsequent transformation. In other media the frequency of evacuation may of course be different (Wolff, 1927).

Sooner or later, the precise moment presumably depending on a variety of conditions, such as the temperature, the state of health of the amoebae, their age,

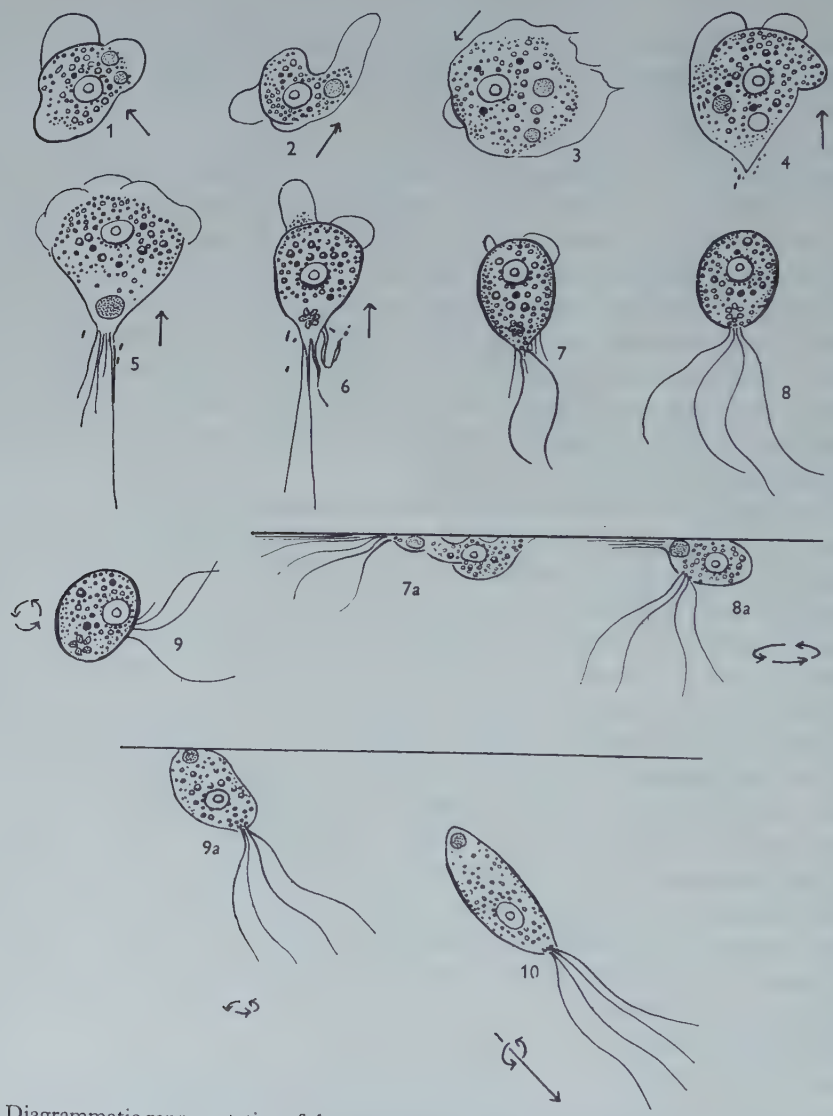

Fig. 1. Diagrammatic representation of the sequence of events as the amoeba changes from the purely amoeboid form (1–3) through the polarized form (4) with filiform pseudopodia (5–6) to acquire flagella (7–8) and then to start spinning (9) and eventually to swim freely away (10). The diagrams 7*a*, 8*a* and 9*a*, are schematic of how the events would appear if the amoebae were seen from the side. They are reconstructions from the normal surface view interpreting events as far as possible in three dimensions.

the number of bacteria present, etc., a short, somewhat thicker, and definite flagellum appears at the posterior end, either among or instead of the filiform pseudopodia (Fig. 1; 7*a*). It is visibly different from the latter; it immediately starts to beat, and appears to be associated with a basal granule, probably derived from the nucleolus (Wilson, 1916). The relationship between the flagellum and the filiform pseudopodia reminds one of the relationship between the true cilia and the finger-like processes which are demonstrable between them by means of the electron microscope in certain ciliated epithelial cells of Metazoa. The one is a special and, in this case, a newly developed structure, the other is a modification of the cell surface.

More often than not this amoeba does not produce a single flagellum but a cluster of three or four, most commonly the latter (Fig. 1; 8, 8*a*). At this time the organism is, as usually observed, lying in contact with the glass of the cover-slip (Fig. 1; 7*a*), though the contact is not a uniform one and this amoeba, like others, may creep on leg-like processes with the 'main body' held somewhat away from the surface. But from now onwards the cell begins to pull itself together into a more compact structure, becoming roughly spherical or ovoid in outline, and while this is happening the point of emergence of the flagella which originated at the posterior end, near the contractile vacuole, tends to move away, over the surface, so that the flagella now beat freely into the medium (fig. 1; 8*a*, 9*a*). The contractile vacuole, however, remains roughly where it was, so that the two organelles become widely separated from each other. A similar reversal of polarity has been noted by Hollande (1942) in *Tetramitus* and also by Martin & Lewin (1914) in *Vahlkampfia.*

As the contractile vacuole fills and enlarges in each cycle the flagella have been observed to beat progressively more slowly and the beat to cease altogether as the vacuole collapses. Then after a short pause the beat starts up again, at an enhanced frequency at first, but it soon settles down once more to a steady rhythm till the cycle is resumed when the contractile vacuole is next ready to discharge. Presumably some change spreads over the surface of the cell as the vacuole breaks through it, and this change involves the flagellar apparatus also.

As the flagella move away from the area of the contractile vacuole and the cell becomes more spherical or ovoid the mean frequency of the beat increases and the cell begins to spin, slowly at first, and then with increasing speed and with wider revolutionary as well as more vigorous rotary movements, till, quite suddenly, it breaks the contact with the glass and swims freely away (Fig. 1; 10). The last point of the attachment seems to correspond to the uroid region, so that as the organism swims away the flagella lead and the contractile vacuole brings up the rear. The revolutionary movements about a fixed point suggest that the attachment to the glass at the end may be due to an extremely fine strand of protoplasm, and certainly in some electron-microscope pictures taken of amoebae (by the kindness of Dr J. R. G Bradfield) at a stage when many of them were becoming flagellate, several amoebae (with flagella) show an extremely fine long thread extending from the posterior end (Fig. 2). Similar thread-like structures have also been observed between amoebae which have been closely associated with each other and then move apart.

A stickiness between amoebae, which may be somewhat similar, has been observed by Wilson (1916).

The change from the amoeboid to the flagellate form can take place in about 20 min. but this time is naturally very variable and, as already mentioned, it may occur at almost any interval between 1 and 24 hr. after the stimulus for it has been applied. The change not only involves the morphological change between the amoeboid and the free-swimming flagellate form, but also is accompanied by the appearance or establishment of a definite cell polarity. Probably also a change in the method of feeding is involved, though on this last point there is still much to be learnt. Some species (e.g. *Tetramitus rostratus*) develop a definite cytostome in the flagellate form (Bunting, 1926), but this has not been observed in *Naegleria*.

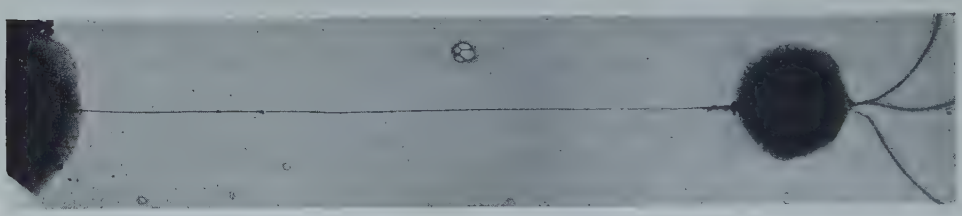

Fig. 2. An electron-microscope photograph (× 5000) of a single amoeba after it has acquired three flagella but is attached to another amoeba by an extremely fine thread. Note the 'flimmer' on the flagella. (Photograph by Dr J. R. G. Bradfield.)

In the experimental conditions which have been maintained in the present series of observations the flagellate phase does not last for very long and after about 24 hr. of free swimming the majority of the organisms begin to settle down as amoebae again. The conditions determining this have not yet been investigated though mechanical agitation may be a factor (Alexeieff, 1924; Wherry, 1913; Wilson, 1916).

In the introduction, emphasis was laid on the possible significance of the change from the phagocytic amoeboid form to the free-swimming flagellate in relation to animalization and vegetalization in embryos. While this may be so there is, of course, one very fundamental difference between free-living Protozoa and the cells of even the simplest metazoan. In the latter case the cells stick together and so form a coherent colony. In other words, the formation of an epithelium of adherent cells is a step which has to be taken by any unicellular organism before it can become colonial. Whether one considers that metazoan colonies have arisen by the coherence of unicellular organisms after division, as the result of random contacts among adhesive cells, or whether one thinks that they have arisen by the ultimate subdivision of the cytoplasm of a large multinucleate organism, in the end the problem remains the same; namely, how and why do the cells remain combined as a colony? This adhesiveness between cells is fundamental to the establishment of a stable metazoan organism. *N. gruberi* normally shows few signs of adhesiveness among its cells either in the amoeboid or in the flagellate form, though when the

cells encyst these cysts usually stick together in clusters. However, one observation on the otherwise amoeboid form may be worth recording. When a drop of a thick suspension of amoebae, carefully washed so as to be nearly free of bacteria, etc., is dropped on to the surface of an agar plate in a Petri dish, the cells generally spread out quickly. On one or two occasions, however, they have formed a continuous sheet in which the cells have taken up particular positions and almost ceased to move relative to each other. Normally they creep freely over each other. When such a sheet of relatively static cells was fixed in mercuric chloride, which is a very suitable fixative for amoebae, the cells apparently remained adherent except in a few places where the sheet as a whole cracked across, presumably due to the shrinkage. The fixed material certainly gave the impression that the sheets formed in this way were composed of cells which had acquired the property of adhering to each other. Other observers have also noted a tendency for the cells to stick together under some conditions (Bunting, 1926; Wilson, 1916) and the formation of the sticky threads mentioned above may be connected with the phenomenon.

How this behaviour is related to the drying up of the colony or to the change to the cyst form has not yet been investigated. Among higher organisms it is very rarely that living cells can be found in direct contact with air, uncovered by a fluid film; thus it may be that the amoebae are also adversely affected by this condition and change their behaviour accordingly.

It appears therefore that in *N. gruberi* we have an organism which can exist at different times as isolated amoebae without definite polarity, as isolated amoebae with an antero-posterior axis, as isolated and polarized flagellates, as cysts, and possibly also as a coherent epithelium. In other words, this single organism shows at least three of the main characteristics of the cells of primitive metazoan larval forms. In the amoebae these states exist at different times; in the metazoan larva they exist simultaneously but are partly distributed in space. In the amoebae the states are certainly interchangeable, and they may often be so in the more primitive Metazoa and their larvae, though, in general, differentiation of cells among the Metazoa is well known to become progressively less and less a reversible process as the evolutionary complexity of the organisms increases.

PHYSIOLOGICAL

An adequate stimulus for the production of the flagellate form is to place the amoeboid form in distilled water. The first problem to be solved, therefore, concerns the means by which the distilled water exerts its effects. Some of the possibilities may, for convenience, be listed. The subdivisions are arbitrary and obviously some of the mechanisms are closely connected with one another. The list will, however, help to bring some order into the varied aspects of the problem.

(1) Mechanical effects (Alexeieff, 1924; Wilson, 1916).

(*a*) Absence of contact with a wetable surface.

(*b*) Absence of proximity to, or of contacts with, neighbouring cells.

(*c*) Mechanical effects of currents in the fluid, etc.

(2) Changes in gaseous exchange.

(*a*) Oxygen (Hollande, 1942; Wherry, 1913).

(*b*) CO_2.

(3) Physicochemical changes.

(*a*) Osmotic pressure (Hollande, 1942; Whitmore, 1911).

(*b*) Hydrogen-ion concentration (Hollande, 1942).

(*c*) Loss of essential ions (Hollande, 1942; Rafalko, 1951; Wasielewski & Hirschfeld, 1910).

(*d*) Changes in the nature of the cell membrane: *a*, passive; *b*, reactive.

(*e*) Loss of essential organic constituents.

(4) Nutritive effects.

(*a*) Absence of bacteria (Schardinger, 1899).

Some of these possibilities will now be considered.

(1) *Mechanical effects*

(*a*) *Contact with surface*

The acquisition of flagella can be followed on the microscope stage in cells which are undisturbed and which maintain their contact with the glass until they are practically free swimming. It does not, therefore, seem to be necessary for the cells to lose contact with the surface before the change can occur. The change can certainly be initiated while the cells are creeping normally.

(*b*) *Contact with neighbouring cells*

While the stock cultures are often very crowded, the amoebae normally live as more or less isolated individuals on the surface of the agar; nevertheless, in view of the well-known effects of isolation in depressing the activities of single cells of other types, e.g. those of metazoan tissue cultures or of sea-urchin eggs, the mere dilution of the amoebae could be important. That it is not a very important factor is fairly clearly shown by Figs. 3 and 4.

The figures show the effect of seeding varying numbers of amoebae into a constant volume (0·5 ml.) of distilled water on the numbers of flagellates which can be counted at various times after seeding. It seems to be clear that the proportion of amoebae which become flagellate, at least within the limits used (limits which were set by the possibility of obtaining reasonably accurate counts), is nearly independent of the number seeded. There is some tendency for more flagellates to appear in the higher concentrations, but this might well be due to secondary effects, as, for example, the population of bacteria in the cultures or multiplication of the cells (Fig. 4). In most cases the number of flagellates increases progressively and more or less linearly between the second and the seventh hours, after which time the numbers become much more constant, and then finally decline. There is no doubt that the curves, expressing numbers of flagellates against time during the period when the cells are acquiring flagella, would all in the end be more or less S-shaped. In some experiments they are more noticeably so than in others. In these cultures very few flagellates appear before about 2 hr. have elapsed after

seeding; sometimes, especially in winter, this interval is considerably longer. Presumably temperature may be one of the factors affecting the results (Schardinger, 1899) and the observations reported in this paper have all been made at room temperature.

In most of the experiments which are to be described the cultures have been set up in the late morning, about noon, and the flagellates counted in the evening. An obvious error arises in consequence of this, since the rising phase of the curves (Fig. 3) is still in progress at this time, and if there are many tubes to sample and count the interval between the first and the last count may be sufficient to cause a significant difference in the numbers of flagellates recorded, since the cultures thus

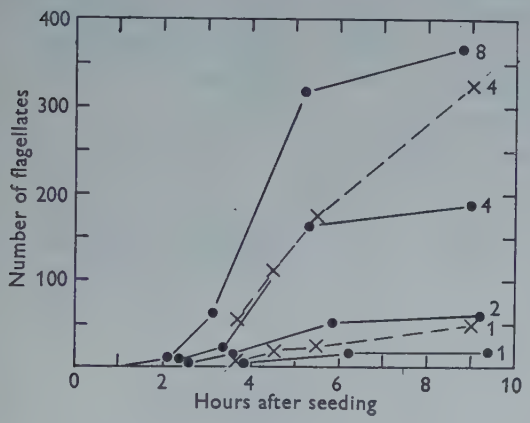

Fig. 3

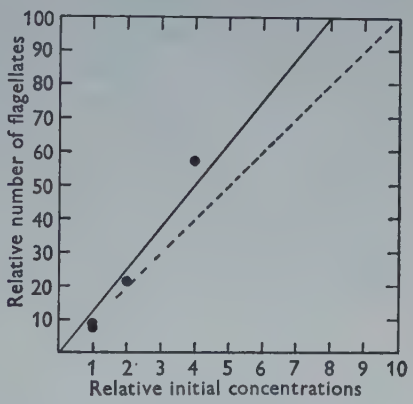

Fig. 4

Fig. 3. Rate of increase in numbers of flagellates related to the initial concentrations. The figure shows the results in two representative experiments. In the first experiment (● — ●) initial concentrations in the proportions 8, 4, 2 and 1 were used. The numbers of flagellates did not increase much after the first 6 hr. and the curves are somewhat S-shaped. In the second (× — — — ×), the numbers of flagellates increased progressively and almost linearly during the same period.

Fig. 4. The relationship between the numbers of flagellates formed and the relative numbers of amoebae inoculated. Combined results of three experiments. The number of flagellates formed is clearly proportional to the number of amoebae inoculated, but as the number of amoebae initially present is increased, so the proportion which becomes flagellate at any one time is somewhat greater than expected. The dotted line shows direct proportionality. This effect could be due to multiplication of amoebae during the experiment.

counted are necessarily at different phases of their activity. In all the later experiments some correction, when necessary, has been made for this by first plotting the numbers found at the actual time of observation and then (using the information plotted in Fig. 3) drawing lines to connect these points to zero at 2 hr. after planting. All counts can then be approximately standardized to a given time, say 6 hr. after seeding, and so made more nearly comparable. This method, which is still only an approximation, was not immediately elaborated, and in many of the earlier experiments recorded here corrections of this kind were not made. This is not considered, however, to vitiate in any way the general results reported, though the detailed figures may be less accurate than one would like.

(*c*) *Effect of currents in medium*

The mechanical effects of currents have not been actually investigated as yet and it will be difficult to dissociate them from the indirect action of currents in accelerating loss of ions, etc. There have been some indications that, in contrast to some previous observations (Alexeieff, 1924), the more the cells are disturbed the more flagellates appear, but so far no quantitative data have been obtained on this point.

(2) *Changes in gaseous exchange*

At first this was thought to be unimportant, since oxygenation is presumably quite adequate for the amoebae on the surface of agar slopes in test-tubes. The same must apply to the removal of CO_2. Nevertheless, when attempts were made to follow the details of flagellum formation under the microscope in contact preparations, i.e. in the fluid contained between the cover-slip and slide, where there is likely to be restricted access of O_2 and also accumulation of CO_2, the numbers of cells which became flagellate in a given time was definitely reduced, as Wherry (1913) had observed, particularly towards the middle of the cover-slip. On the other hand, in experiments in closed tubes with an air space and with a side-arm to contain gas absorber, no effects have been noticed in the number of cells which

Table 1

	With CO_2 absorbent	Without CO_2 absorbent
Total flagellates counted	1577	1426
No. of tubes	42	39
Flagellates per tube	38	37

became flagellate when the O_2 or CO_2 were reduced by absorption in alkaline pyrogallol or in 40% NaOH, respectively. The absence of any effects of absorbing the CO_2 with NaOH is shown in Table 1. As will be seen later, dissolved CO_2 and the bicarbonate ion may be important in connexion with the hydrogen-ion concentration of the medium, and the latter is probably important in its own right.

Obviously the respiratory metabolism of the cells will have to be investigated in much more detail, but until the amoebae can be obtained free from living bacteria and cultured in their absence no great advance can be made in this direction. From the present point of view there is no evidence that conditions of oxygenation or of CO_2 elimination are the determining factors in causing the amoebae to become flagellate, though both may be of subsidiary importance. Some effects in restricting the numbers of flagellates formed which were observed with methylene blue in the medium, and which will be described in more detail in another paper, should perhaps be considered in relation to their bearing on respiration as well as in relation to the context in which they will be discussed.

(3) *Physicochemical changes*

(*a*) *Osmotic pressure*

The effects of osmotic changes, as such, can be assessed by comparing the behaviour of the amoebae in solutions of NaCl, glucose, sucrose, etc., of varying concentrations. M/2·5 solutions of these substances were therefore prepared in distilled water and serial dilutions made from them so that the concentration was halved each time. When these dilutions were seeded with amoebae, and the numbers of organisms estimated at intervals thereafter, it was found that the amoeboid form survived concentrations of M/5 glucose and sucrose for at least 24 hr. In M/2·5 solutions, the amoebae shrivelled and rounded off, though a few might remain active for a time. In sodium chloride, the highest concentration for stability was M/10 with some temporary survival in M/5 (Fig. 5). These figures, therefore, suggest

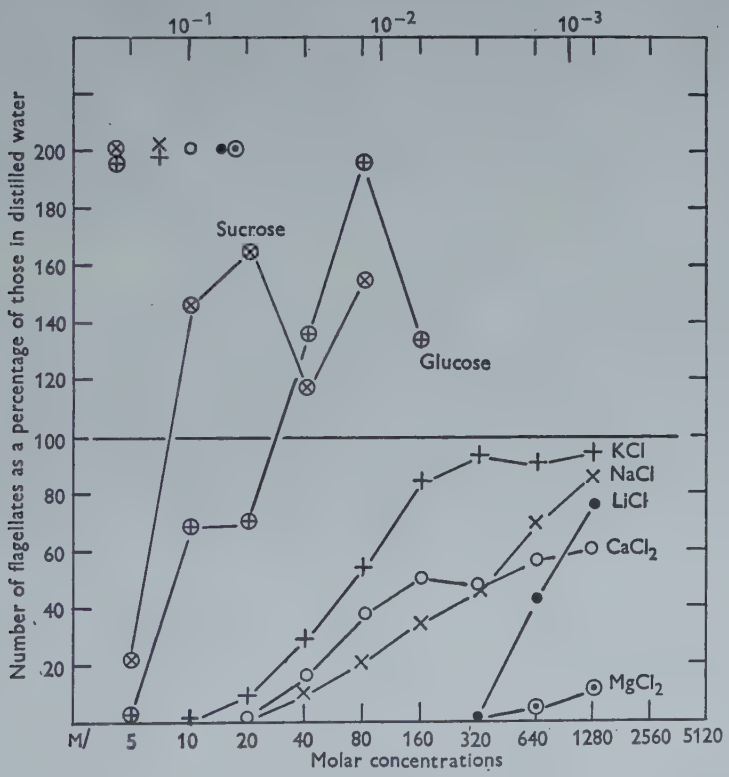

Fig. 5. The effects of sucrose, glucose and certain cations on the behaviour of the amoebae. The curves represent the effects of different molar concentrations of the chlorides on the number of flagellates which form as compared with the number which form in parallel experiments in distilled water alone (standardized at 100). Although the actual number which form in water is unaccountably variable, it does provide a standard by which different experiments, with different cultures of amoebae, can be compared. All the salt solutions used depress the formation of the flagellate form, sucrose and glucose appear to encourage it, but this may be simply because they act as food, so that the amoebae remain in better condition, perhaps even multiplying. The maximum concentrations at which normal amoebae were observed to be active are shown by the appropriate symbols in the top left corner.

that osmotic effects become too powerful for the amoeboid form at about these concentrations. The NaCl, as might be expected because of its ionization, was about twice as potent as the unionized sugars, and $CaCl_2$ and $MgCl_2$ still more so. In a preliminary experiment with urea and hexamine (hexamethylenetetramine), amoebae survived in an active form in M/2·5 in each case. The osmotic effects here can be presumed to be less, since these substances probably penetrate into the cell rather easily.

In glucose, sucrose, urea and hexamine solutions the flagellate form appeared in all concentrations up to those which were lethal to the amoeboid form, though the numbers appearing showed (except in the sucrose solutions) a tendency to fall off at the highest concentrations, thus indicating that the flagellate form is not so stable as the amoeboid form and is more easily depressed, a fact which has been abundantly confirmed throughout all the observations recorded in this paper.

In sodium chloride solutions, on the other hand, the flagellate form became less and less numerous as the concentration was increased above M/1280, and flagellates were very rare in concentrations greater than M/40.

These observations therefore indicate that osmotic pressure as such is not the determining factor in causing the change from the amoeboid to the flagellate form, though it again may exert its influence, as might be expected, on the activity of the organisms.

(*b*) *Hydrogen-ion concentration*

The amoebae can stand a very wide range of hydrogen-ion concentration; at least they are tolerant of such for a limited time. It must be remembered, however, that the effects reported here are only those observed within the first hours of seeding the amoebae into solutions of varying hydrogen-ion concentration. Nevertheless, it may be stated at once that the hydrogen-ion is not, in itself, the determining agent in causing the assumption of the flagellate form, and indeed the effects of pH are often secondary in other respects to those of the positive and negative ions present in the buffer solutions used. Flagellates may appear at any pH between 5·5 and 10, though there is generally a definite optimum and this optimum depends on the buffer solutions used (Figs. 6, 7). For example, in buffer solutions made by mixing M/160-NaH_2PO_4 and M/160-Na_2HPO_4 (Fig. 6) the maximum lies between pH 6 and 7, and definitely fewer flagellates occur in solutions on the alkaline side of neutrality. On the other hand, in mixtures of M/160-NaH_2PO_4 and M/160-Na_2CO_3 there is a definite maximum between pH 7·5 and 8, and in this solution alkalinity favours the flagellates. In mixtures of lactic acid and sodium carbonate flagellates were abundant on the alkaline side but were entirely absent at pH 6 (Fig. 8). It is thus obvious that while pH, as in all biological systems, is important it does not appear to be the critical factor in relation to the flagellate condition. Similar observations have also been made with other concentrations of the buffer solutions, and it is noticeable that as their molarity is increased so the numbers of flagellates is at first increased and then rapidly decreases again (Table 2). Fig. 7 shows the effects of pH in pure phosphate, and in phosphate-bicarbonate,

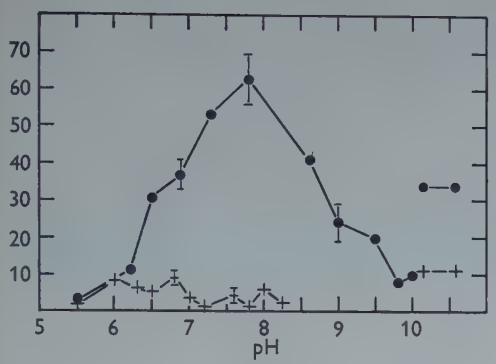

Fig. 6

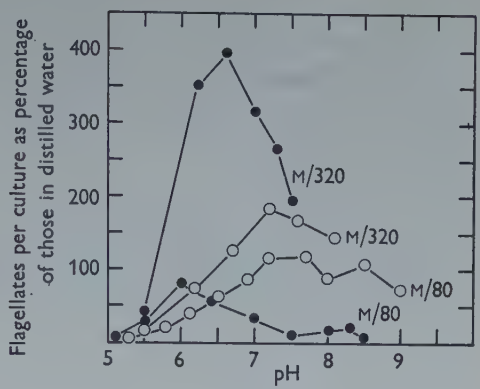

Fig. 7

Fig. 6. A typical experiment showing the effects of pH on the numbers of flagellates formed (each point represents the mean of six tubes) in M/160-NaH_2PO_4+M/160-Na_2CO_3 mixtures (●—●) and also in M/160-NaH_2PO_4+M/160-Na_2HPO_4 mixtures (+ — +). The short lines represent the numbers which became flagellate in the relevant distilled water controls. The vertical bars represent twice the standard error of the mean.

Fig. 7. Graphs showing the influence of certain anions on the pH optima for the acquisition of flagella, at two different concentrations. Phosphate buffers produce their maxima on the acid side and phosphate-bicarbonate buffers on the alkaline side of neutrality. ●—●, NaH_2PO_4+Na_2HPO_4; ○ — ○ NaH_2PO_4+$NaHCO_3$.

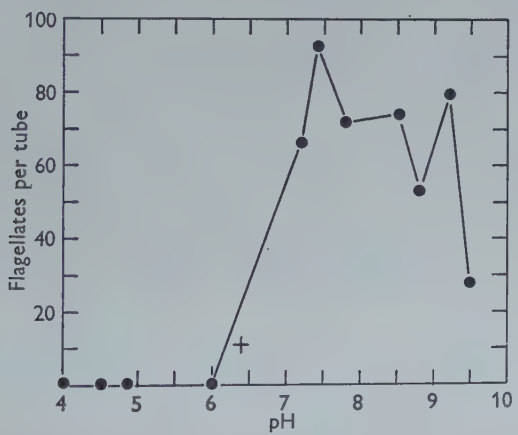

Fig. 8. Graph showing the results in a single experiment with cultures in mixtures of lactic acid and sodium carbonate at different pH values (means of initial and final values determined colorimetrically, and thus approximate only). Note the large numbers of flagellates in the alkaline solutions as compared with those in the distilled water controls (+).

Table 2

Molarity of 1:1 mixture of $NaHCO_3$ and NaH_2PO_4	Relative no. becoming flagellate
0 (distilled water)	100
M/320	316
M/160	228
M/80	180

buffers at M/80 and M/320. With higher concentrations the pH optimum for the flagellate form seems to creep towards the acid side with the purely phosphate buffers, and definitely to the alkaline side with the phosphate-bicarbonate systems. How much the extra sodium contributes to the adverse effects of alkaline phosphates is a moot point.

(*c*) *Loss of essential ions*

In the experiment reported above on the effects of osmotic pressure and of pH various ions have been used to produce the requisite solutions, and it is abundantly clear that the nature of these ions is a matter of no little importance. The questions, in addition to those of osmotic pressure and pH, which are raised by the effects of various ions on the behaviour of these amoebae are, of course, numerous and diverse and could be tackled in a variety of ways. On the whole, biological systems are mostly dependent on a proper balance of certain inorganic salts in their environment. Na^+, K^+, Ca^{2+}, Mg^{2+} are important cations, Cl', PO_4''', HCO'_3, and lactate are often important anions. A beginning has therefore been made in studying the response of *Naegleria* to some of these biologically important ions in order to see which of them act as determining factors in altering the 'phase' of the amoebae, i.e. from the amoeboid phase to the flagellate phase or vice versa. M/5 solutions of various salts have been made up and, from these, serial dilutions ($\times 2$) have been made and their effects on the amoebae studied. In some cases, the serial dilution modifies the hydrogen-ion concentration, so that the effects of this have to be disentangled from those caused by the ions under investigation.

A comparison has been made of the various positive ions by making serial dilutions of the chlorides. The results are summarized in Fig. 5, which also includes the results of adding lithium chloride; the interesting actions of the lithium ion will be discussed below.

All the ions suppress the formation of flagella to some extent as compared with solutions of sugars or with distilled water. There is not a very great difference between K^+, Na^+ and Ca^{2+}; they all begin to reduce the numbers of flagellates at concentrations greater than M/1280, the K^+ ion perhaps being the least depressing. On the other hand, magnesium is very effective in maintaining the amoeboid form and suppressing the change into the flagellate condition. Even in concentrations as low as M/1280 there is a very pronounced reduction in the number of flagellates as compared with that found in the distilled water controls. There is some suggestion that this action of Mg^{2+} is one of delaying the onset of the change to the flagellate form, since in a few cultures examined after 24 hr. flagellates were found in considerable numbers. The amoeboid form persists in an active state in Mg^{2+} concentrations up to M/20. This limit is somewhat lower than those for the monovalent salts (Fig. 5), but there is obviously a large range of concentrations in which the amoeboid form is stable and in which the tendency to become flagellate is suppressed. A somewhat similar state of affairs is found with lithium, but whereas the effects of magnesium gradually decrease with decreasing concentration, the effects of lithium seem to come on more suddenly at about M/1280. Another point about

lithium is that at the highest concentrations, before it becomes lethal (i.e. about M/20), it causes the amoebae to assume very characteristic shapes as shown in Fig. 9. The long cylindrical process is somewhat reminiscent of the change of form which frequently occurs in the choanocytes of sponges (*Sycon*) when these organisms are dissociated into sea water. The significance of the change is obscure.

From the results shown in Fig. 5, it seems to be fairly clear that the presence of the usual biologically active ions in the environment opposes the formation of flagella, so that it would appear likely that the flagellate phase is perhaps an adaptive form to counteract loss of these ions, and vice versa, that the stability of the amoeboid form depends on their presence in the medium.

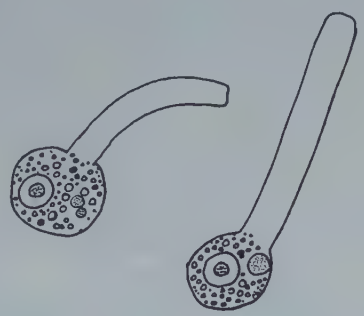

Fig. 9. Diagrams to illustrate the forms commonly assumed by *Naegleria gruberi* in solutions of lithium salts at about M/20 concentration.

In addition to those mentioned above, because of their influence on pH, the effects of some anions have also been studied by using serial dilutions of various sodium salts. The results are plotted in Fig. 10. Sodium sulphate and chloride tend to stabilize the amoeboid form and to prevent the change towards the flagellate form. The sulphate ion is particularly effective in this way, but it also proves to be somewhat toxic to the amoeboid form. On the other hand, the lactate, bicarbonate and phosphate ions all favour the production of the flagellate form when present in concentrations between M/80 and M/5120. The effects of the last two ions have, of course, to be considered in relation to the pH effects, since dilution of these salts lowers the pH. As we have seen above, alkalinity in phosphate solutions is not favourable to the flagellate form, so the beneficial effects of phosphate in the flagellate transformation are confined to very dilute solutions. On the other hand, quite high concentrations of bicarbonate solutions are consistent with the change to the flagellate form.

Some interesting results were obtained when cultures were made by adding the various salts to a buffer solution (pH approx. 8·0) consisting of:

$NaHCO_3$	0·84g. (0.01M)
KH_2PO_4	0·23g. (0·00167M)
Distilled water	1000 cc.

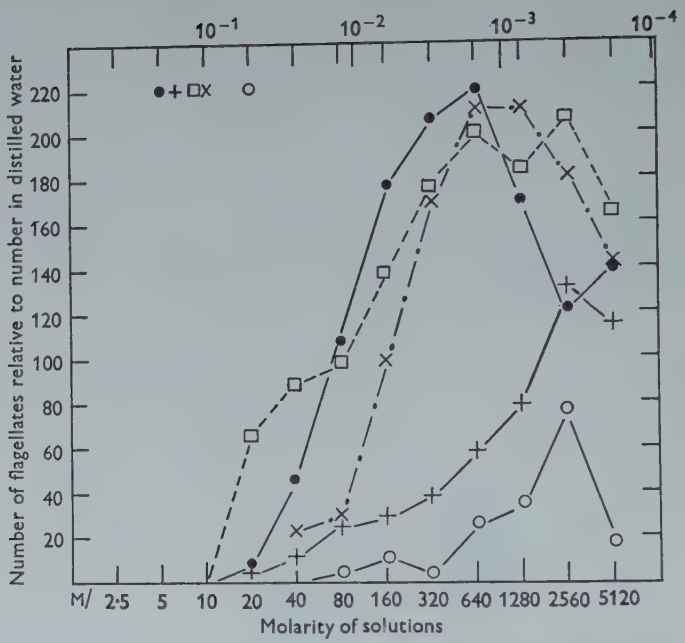

Fig. 10. The effects of different concentrations of anions in favouring or hindering the assumption of the flagellate form. The ordinates express the numbers of flagellates formed in the different concentrations of sodium salts relative to the number formed in distilled water, which was standardized at 100. The isolated points (top left) illustrate the molarities at which amoeboid (as opposed to flagellate) activity is suppressed. □ – – – □, sodium bicarbonate; ● — ●, sodium lactate; × — · — ×, di-sodium hydrogen phosphate; + — +, sodium chloride; ○ — ○, sodium sulphate.

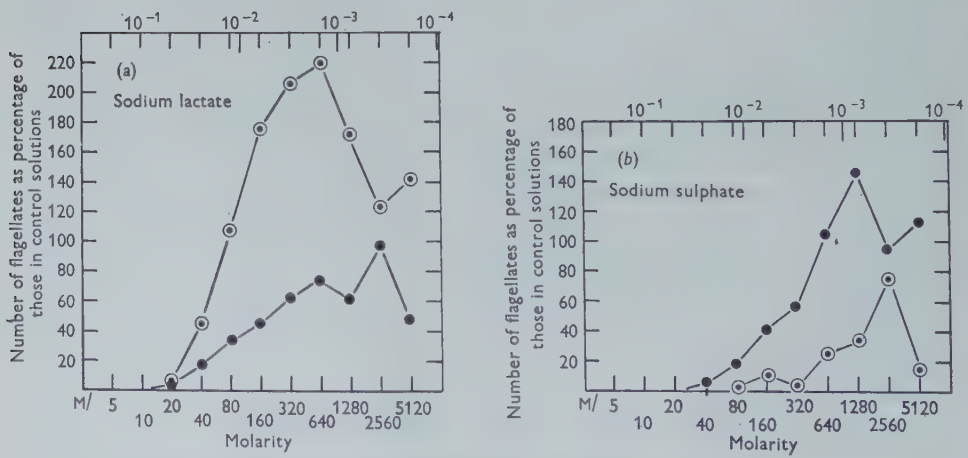

Fig. 11. Diagrams showing the difference in the action of sodium lactate and sodium sulphate when these salts are applied in distilled water (⊙ — ⊙) or in a bicarbonate-phosphate buffer (pH 8·0 approx., see p. 599) (● — ●). In each case the ordinates represent the numbers of flagellates formed as percentages of those formed in the water or buffer solution alone.

It was then found that there was much less difference between the effects of the anions. Sodium sulphate became much less inhibitory to the formation of the flagellate type and sodium lactate less stimulating; that is to say, there was no evidence that lactate increased the numbers of flagellates over and above that found in the buffer solution alone, which was, of course, greater than in water alone owing to the presence of bicarbonate (Fig. 11). There must thus, as in other situations, be considerable interaction between the ions in determining how the cells behave. It would seem that the bicarbonate, the lactate and, in dilute solutions, the phosphate ion all have similar effects; all encourage the formation of the flagellate form while the sulphate ion, on the other hand, depresses the metamorphosis, but its action is

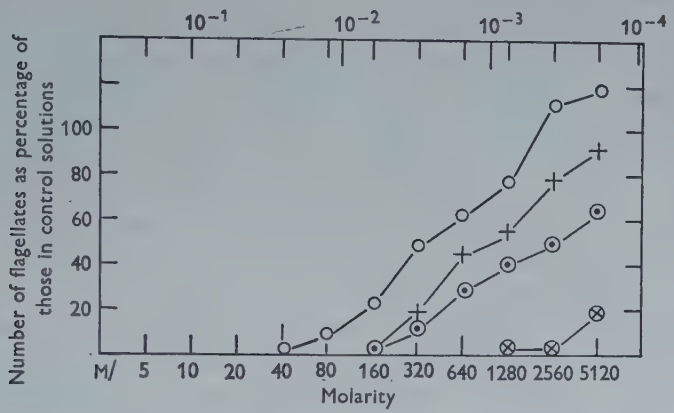

Fig. 12. Diagram (cf. Fig. 11) showing the action of lithium salts when applied either in distilled water, or in bicarbonate-phosphate buffer (see pH 8·0 approx., see p. 599). ○ — ○, Lithium lactate in buffer; ⊙ — ⊙, lithium lactate in water; + — +, lithium sulphate in buffer; ⊗ — ⊗, lithium sulphate in water.

largely annulled by the presence of bicarbonate. When lithium salts were studied in this way, instead of the sodium salts, it was again found that the sulphate became less inhibitory to the change to the flagellate form when it was applied in bicarbonate buffer solution; but so also did the lactate, in contrast to the behaviour of sodium lactate (Fig. 12). In buffer solution there was indeed little to choose between the actions of lithium chloride, lactate and sulphate; all were less inhibitory than they were when applied in water and the difference in the case of the sulphate was very large.

DISCUSSION

The capacity of *Naegleria gruberi* to change from one form of cell function to another in response to changes in its environment offers a splendid opportunity to investigate in isolation one of the types of change in cellular activity which occurs among metazoan cells both during embryonic development and also in cases of metaplasia later in life.

On the addition of water, *Naegleria* changes from being a more or less non-polar cell which creeps about, mostly by means of lobose pseudopodia and without any clear orientation, to become a highly polarized cell, progressing by means of flagella.

Almost nothing is known of the nature of this change. Since it occurs when the cells are placed in water it may be an attempt by the cell to counteract the loss of important constituents. It seems likely enough that in distilled water the amoeba should lose salts, and that to counteract this loss it may assume the flagellate form, perhaps with a different mechanism for maintaining water and salt balance, and almost certainly with a modified cell surface. Thus, temporarily or perhaps permanently stabilized, it swims away to find some more suitable environment. From an ecological point of view this may perhaps be considered as an adaptation, in that, when water is abundant in the environment of the amoeba, the animal may benefit from it to extend the range of the species more quickly than it could do in the amoeboid form.

Magnesium, which in many cells is known to decrease permeability, may act by checking this loss of essential ions, and so rendering unnecessary the compensating change of form. Bicarbonate and lactate ions, on the other hand, encourage the metamorphosis and favour the flagellate form. The action of the magnesium ion in stabilizing cell matrices in higher animals and its action in binding proteins and polysaccharides may be significant for the amoeba. The trails of protoplasm and the somewhat glutinous uroid of the polarized amoeba should perhaps be investigated from this point of view.

It was mentioned earlier that the behaviour of *Naegleria* might throw light on the nature of the gradient which exists in many embryos and larvae and which may manifest itself by the larva possessing flagellate cells in the anterior (or animal) half, and cells of a more phagocytic character in the posterior (or vegetal) half. It is well known that this gradient can be upset by the presence of lithium salts, and it is of particular interest therefore to find that lithium salts are among the effective agents which favour the amoeboid form of *N. gruberi* and suppress the flagellate form. It is true that on many marine embryos the lithium salts are effective in the presence of sea water, while in the case of *Naegleria* the presence of even small amounts of sodium bicarbonate and potassium phosphate lessens their effect; nevertheless, the action of lithium salts is similar in the two cases in that it suppresses the flagellate form. It would be interesting to know more of the effects, if any, of magnesium salts on the vegetalization of embryos.

The two other changes of form which *Naegleria* shows in response to changes in the environment may also be important in relation to the formation of colonies of cells and the early differentiation of Metazoa. Under unfavourable conditions of food supply (e.g. after about 10 days on the agar-Lemco culture slope) *Naegleria* encysts, and the cysts have that capacity for sticking together which is such a necessary preliminary to colonial organization. A similar tendency for the cells to stick together has also been shown by the amoeboid form itself when plated out in large numbers on to the relatively dry surface of an agar gel in a Petri dish; under these conditions the amoebae were observed to form what appeared to be an epithelial sheet.

It thus appears that *N. gruberi* may prove to be an important and readily available organism for the experimental study of some of the primary manifestations of cellular differentiation and perhaps also of colony formation.

SUMMARY

1. When placed in distilled water *Naegleria gruberi* changes from an amoeboid organism, with little evidence of polarity, to a highly polarized free-swimming flagellate. The details of this metamorphosis are described. The change is reversible.

2. Alteration of osmotic pressure is not in itself the direct cause of the metamorphosis, though the loss of certain ions is clearly important.

3. The metamorphosis is favoured by the presence of low concentrations (less than M/80) of sodium bicarbonate, sodium lactate and sodium phosphate.

4. The flagellate form probably occurs most frequently in conditions of neutrality; but, in the presence of phosphate, acid conditions tend to be more favourable to the flagellate form, while in the presence of bicarbonate the optimum pH is nearer pH 8·0.

5. The metamorphosis to the flagellate form is suppressed by a variety of agents including lithium salts, magnesium chloride and the sulphate ion under some conditions. These all act at concentrations which leave the amoeboid form in full activity. In some cases their action is decreased by the presence of bicarbonate in the medium.

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THE RESPONSES OF *HETEROXENIA* (ALCYONARIA) TO STIMULATION AND TO SOME INORGANIC IONS

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The Red Sea Alcyonarian *Heteroxenia fuscescens* (Ehrb.) consists of a pinkish brown fleshy syndete up to 5 cm. in length, firmly attached to a stone or dead coral, from which grows a mass of autozooids with columns 2–4 cm. long and 2–3 mm. in diameter (Fig. 1). The first physiological observations on a xenid were made by Keller (1883) on a brown species from Suez that he considered to be *H. fuscescens*. He described the rhythmical movements in which the tentacles are clapped together (Fig 2A). The zooids beat independently of one another, and the two halves of a disk, split longitudinally, continued to beat as before but independently. Leaning on the Hertwigs' (1879) statement that the nervous system of actinians is best developed in the disk, Keller came to the conclusion that there is here a diffusely spread rhythmical centre. He also pointed out the resemblance to the beat of medusae.

Krukenberg (1887) worked on colonies which he identified as *Xenia umbellata* at Massawa and *H. fuscescens* at Suez. From the results of operations on the disk he inferred that a rhythmical nerve centre was located in the peristomial region. He also studied the transmission of excitation. A weak mechanical stimulation of a tentacle produced a response of only one tentacle, which curled over the mouth. Following stronger stimulation the whole crown of tentacles temporarily folded together as in Fig. 2B, and neighbouring zooids behaved similarly. In the present paper this reaction is called a 'spasm'. Krukenberg inferred a 'ganglionic nerve net', best developed in the disk and tentacles and condemned Keller's conclusions (*a*) that the nervous centres of the polyps are entirely independent, and (*b*) that the rhythm is unaffected by external stimuli. He finally concludes that it seems unlikely that the rhythm corresponds to that of medusae.

A few other authors refer to the rhythmical beating of xenids; for example, Saville Kent (1893, p. 197) says 'all the eight tentacles move synchronously, opening out and contracting in a continuous measured rhythm', and Hiro (1937) gives the rhythm of *H. elizabethae* as 30/min. for large and 37/min. for small colonies. Hiro describes the spasm and agrees with Keller, *contra* Krukenberg, that other zooids are not affected when one is stimulated or even cut off. In his review of the Red Sea xenids, Gohar (1940) added some physiological observations. He found a temporary acceleration of the rhythm when small inert particles fell upon the tentacles and observed the folding of a single tentacle while the rest continued their rhythm. On the transmission between zooids he agrees with Krukenberg.

In the present paper the rhythmical beat and the co-ordinated spasm provide a means of studying the propagation of excitation. The intimate mechanisms of the beat and of the spasm are unknown, but it will be shown that the first has much in common with the rhythm of medusae, and that the second has an absolute refractory period of 0·08 sec.

Fig. 1. A colony of *Heteroxenia fuscescens* (Ehrb.).

MATERIALS AND METHODS

Naturally growing colonies of *Heteroxenia fuscescens* were examined by diving at Ghardaqa, Red Sea, and specimens were removed with their substrate. They thrive in an outside tank but soon disintegrate if kept out of the light. Colonies may not be torn from their substrate, for any small damage to the syndete releases the internal water pressure and the circulation through the colony is interrupted. For the same reason experiments which require section through the colony are impracticable. Details of the care of xenids are given by Gohar (1940).

The arrangements for electrical stimulation and measurement of the refractory period were as figured by Pantin (1935), with a condenser shock as stimulus. With this arrangement the form of the stimulus depends on its intensity, but the simple circuit is a great advantage.

The zooids do not all beat at the same rate, and for any one of them the interval between beats is not quite constant. Accordingly, an arbitrary but convenient measurement was employed; the time for twenty (or for thirty) beats was taken for a number of zooids. This is given as an average for ten zooids with its standard deviation between zooids.

Solutions of potassium sulphate and of sodium, magnesium and calcium chlorides were prepared isotonic with the local sea water, taken as 120% normal sea water.

QUALITATIVE OBSERVATIONS

In the normal rhythm the eight tentacles are bent stiffly and symmetrically inwards by muscles on their upper sides. The contraction is a single co-ordinated twitch which brings the tentacles together (Fig. 2A). The relaxation suggests that they spring back under elastic forces. This continues day and night at 30–45/min. It is not co-ordinated between zooids and the continuous activity is a striking sight.

A different movement is the maintained spasm of one or more tentacles. Apparently the same muscles contract as in the beat but with a much greater intensity

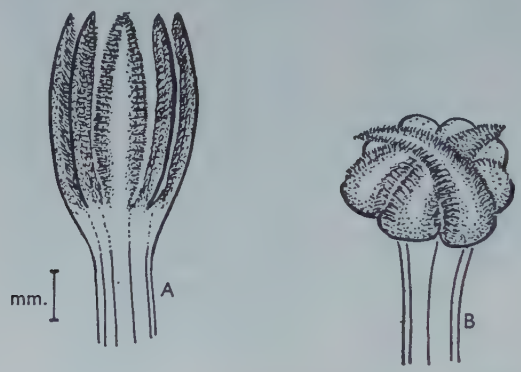

Fig. 2. A single autozooid; A, contracted in a beat; B, contracted in a spasm.

(Figs. 3B, C, D). This can occur in response to a touch, but sometimes an occasional tentacle bent inwards spontaneously while the others beat unaffected. When lightly touched with a needle a tentacle will sometimes respond alone but usually the excitation spreads round the disk and the eight tentacles contract together (Fig. 2B). In most colonies the spasm affects all zooids when any part is stimulated strongly. The transmission across the syndete is not prevented by a cut which excludes transmission in the superficial layer and is therefore not ectodermal.

A difference in the anatomical arrangement of two conducting systems is shown as follows. An autozooid disk is split vertically and allowed to recover from the shock; each half now beats independently, as Keller found, but a pinch to one half produces a spasm of *both* halves. The responses were also examined for any interaction between the rhythm and the spasm. It sometimes happened spontaneously that two opposite tentacles would bend inwards by chance at the same time (Fig. 3C). They would remain bent for many beats, but the groups on either side would continue in perfect co-ordination. Further, six tentacles could respond independently to delicate touches while two were unaffected (Fig. 3D). These two continued in unison, though not necessarily regularly, until the others again joined their rhythm. These simple qualitative observations were repeated many times.

The rate of the rhythm is found to be slower in severed parts than in whole zooids. It is also more variable, both between parts and from time to time in single parts. These effects are more marked the smaller the severed piece (Fig. 4). This is interpreted by considering that the origin of the rhythm lies in a population of

units, each with its own rhythm and that the rate of the whole is the rate of the fastest. The simple picture is not entirely borne out by experiment, for, if a zooid disk is divided into eight segments, the minimum interval between beats is always greater than in the original zooid. The same result is usually found with medusae, and implies the existence of excitation other than that within the rhythmical system.

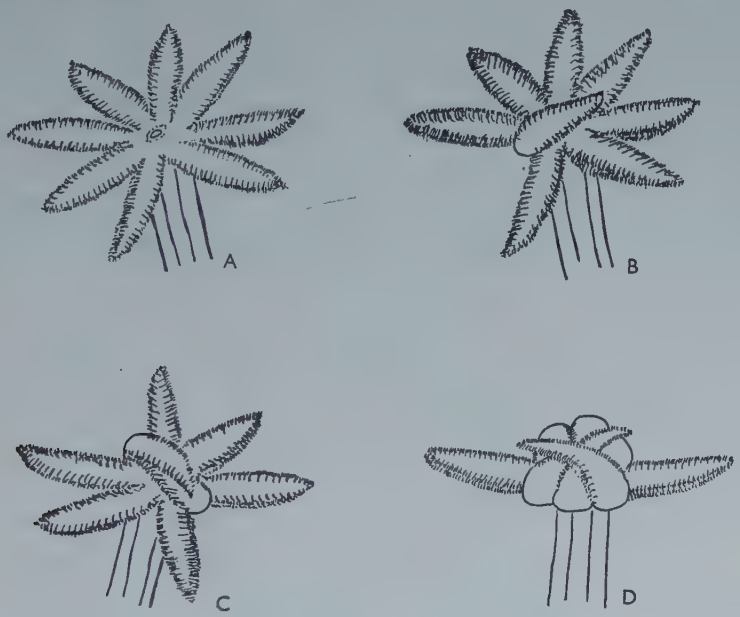

Fig. 3. A single autozooid; A, relaxed; B, C, D, in a partial spasm as described in the text.

ELECTRICAL STIMULATION

It proved impossible to introduce an extrasystole into the rhythm by a single shock because a spasm always intervened. The response of an isolated tentacle to a weak shock is a small jerk. An increase or a decrease of the rate of the rhythm may follow weak electrical stimulation. After a spasm the normal rhythm is resumed, but *Xenia macrospiculata* Gohar takes several beats to regain its original frequency after a spasm.

A single shock above threshold applied on the head of a zooid produces a spasm that normally lasts 6–9 sec. There is no retraction of the column. The response is independent of the strength of the stimulus except for a small stimulus to a tentacle, which may then contract alone; at high intensities spasms of 15–30 sec. were regularly found.

Two shocks at the same point on the disk at an interval of 0·1–2 sec. produce a similar spasm, but now the column of the zooid partially withdraws; neighbouring zooids are not usually affected. With three shocks, intervals 1 sec., several neighbouring polyps go into a spasm for 6–9 sec., and the stimulated one retracts its column. Four shocks, intervals 1 sec., produce a spasm of about thirty zooids over an area 3×3 cm. for 6–9 sec., and perhaps this time a few retract in the neighbourhood

of the stimulus. With a greater number of shocks, or with three or four shocks at $\frac{1}{2}$ sec. intervals, all zooids show the spasm, but again the partial retraction of the column is limited to those around the one stimulated.

The above results were characteristic of a particular group of colonies when first stimulated, and other colonies differed as mentioned below. When experiments are repeated after 2 min. the spasm is more easily propagated over the colony than before. For example, two shocks, 1 sec. apart, now produce a spasm of all the colony.

With the electrode on the surface on the sydnete, or buried in it, a single shock has no effect. Two shocks 1 sec. apart can produce a spasm of all zooids but usually three or four are necessary. Two shocks, interval 4 sec., have no effect. At a stimulus interval of 3 sec. a curious effect is obtained. The stimulator is left running at this frequency; a few neighbouring zooids go into a spasm and eventually retract, but over the rest of the colony only a few show a spasm. Though the rhythm con-

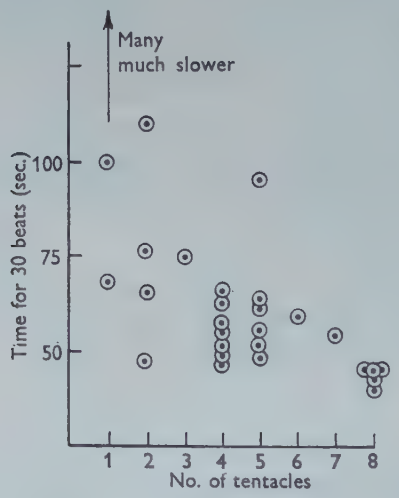

Fig. 4. The frequency of the rhythm of isolated segments of the autozooid disk.

tinues, there is a general appearance of 'sleepiness' in all the zooids; they are on the brink of going into a spasm, and do so if the stimulus frequency is increased. The exact limit varies between colonies and is increased by continued stimulation. In one colony stimulation at 40/min. produced the sleepiness but at 45/min. there was an immediate co-ordinated spasm and a retraction of all zooids. However, during 2 min. stimulation at 45/min. or even 50/min. the polyps slowly begin to beat again, though not at maximum frequency and the tentacles tend to be half contracted.

If the relatively long-term effects of stimulation are ignored, the transmission of excitation shows two characteristics. First, excitation is transmitted less readily from a stimulated disk than from a point on the syndete. Secondly, the number of *additional* zooids affected increases rapidly with each successive stimulus and there is a definite number of stimuli which initiate a wave over the whole colony. This is

characteristic of some alcyonarian colonies in contrast to madreporarian and zooanthid colonies. With stimuli at, say, 1/sec. there is at first only a local spread of excitation, and in some alcyonarians, *Lobophytum*, for example, 10–12 stimuli may be required before the wave of zooid retraction suddenly begins to spread over the whole colony. On account of their small size the colonies of xenids are not suitable to demonstrate the details of co-ordination over the colony, and all the zooids contract before the third or fourth shock.

In certain colonies with large zooids a single shock on the column wall of a zooid produced no visible result, but a second shock, interval 1 sec., initiated a spasm in this zooid alone. In other colonies, usually less developed and with smaller zooids, a single shock on the column produced a spasm in that one zooid and a second shock (interval 1 sec.) produced a spasm in neighbouring zooids. In another series of experiments a small colony was used which responded to the second of a pair of stimuli, interval 1 sec., on the column or syndete by a co-ordinated spasm of all zooids. The second stimulus was then applied at a point on the syndete as far as possible (i.e. 5 cm.) from the first. The second stimulus now produced no effect. However, when the second stimulus was applied 2 cm. away from the first a co-ordinated spasm followed. Such results varied greatly between colonies, but most experiments with one group of colonies showed that the second stimulus must be within 2 cm. of the first to produce a wave of spasm; but in some specimens two stimuli produced such a wave only if applied within a few millimetres, and again in many colonies two stimuli produced no result at all.

The strange result of Keller, who found no co-ordination of the spasm over the colony, could possibly be explained by the following observation. A colony from a shady place in 3 m. of water at low tide had very large zooids 6–8 cm. long with an 'etiolated' appearance. Strong shocks on the syndete at $\frac{1}{2}$ sec. intervals produced no spasm. Stimulated disks showed the spasm as usual, but no transmission to other zooids could be induced.

A refractory period of the 'colony' (as distinct from the 'disk') conducting system was measured in some colonies, which responded by a spasm of at least a few zooids to the *second* of a pair of shocks applied on the syndete. The apparatus was as figured in Pantin (1935), with a rotary arm and mercury cups to provide the variable interval. No result followed a single stimulus. For several colonies, with stimulus strength at least four times the threshold, there was no response to the second of the pair of stimuli at an interval less than 0·08 sec., but with intervals greater than 0·1 sec. a spasm of some zooids always followed.

EFFECTS OF IONS

Potassium

The effect of excess potassium ions on *Heteroxenia* is twofold. First, there is a slowing of the rhythm, which stops after 50 min. immersion in sea water containing 5% of isotonic K_2SO_4. Secondly, the responses of the tentacles, the sensitivity of the colony surface to touch and the co-ordination of the spasm over the colony progressively

disappear. The final result is a *relaxed*, paralysed colony, effectively narcotized, and turgid as a result of the hyperactivity of the cilia. As an example, ten counts of one colony initially gave an average of twenty beats in $29{\cdot}7 \pm 0{\cdot}9$ sec. S.D. Isotonic potassium sulphate was gradually added up to 5% of the total volume. For 20 min. there was little effect, but after 30 min. many zooids took 40 sec. for twenty beats and after 40 min. the few that remained active took more than 60 sec. for twenty beats. Some examples showed a slight initial acceleration. If the potassium sulphate is quickly added the chemical stimulation produces temporary spasms.

In the relaxed and insensitive condition produced by excess potassium ions neither the longitudinal muscles nor the tentacles will respond to electrical stimuli at 1/sec. The circular muscle of the column, however, will still contract very slowly. This produces an interesting result, for now the sphincter formed by a contracted band of circular muscle isolates hydraulically the distal part of the zooid, which shrivels, while the basal part inflates to a sphere. During recovery in normal sea water the shrivelled polyp head recovers its rhythm before the rest of the colony because it is isolated from the reservoir of potassium ions in the fleshy syndete. A series of stimuli now applied to the syndete fails to produce a spasm in this polyp, though the polyp itself responds normally. This shows that excitation which normally co-ordinates the spasm of the colony is also stopped by the excess potassium ions. During recovery the local response of tentacles and the spasm appear before the onset of the rhythm.

Sodium

An excess of sodium ions in the medium first accelerates the rhythm, then, in strong enough solutions, slows it. In one experiment with a slowly beating colony the initial count was ten beats in $22{\cdot}2 \pm 2{\cdot}4$ sec. S.D. Isotonic sodium chloride was added to make 14% of the total volume. After 2 min. the average for ten counts in the next 5 min. was ten beats in $18 \pm 1{\cdot}34$ sec. S.D., and after 25 min. the average for ten counts was ten beats in $26{\cdot}7 \pm 3{\cdot}0$ sec. S.D. In all observations there is first an acceleration but the interval between beats is never less than 1·1 sec. Then, as the sodium concentration in the tissue rises, the zooids become more liable to respond to the slightest touch by a long-sustained spasm, and with mixtures containing more than 10% sodium chloride permanent spasms prevail. This contrasts with the relaxation produced by potassium ions and suggests that sodium ions are not directly slowing the rhythm. No effect on the co-ordination of the spasm over the whole colony was found.

Magnesium

Excess magnesium ions anaesthetize *Heteroxenia*. Isotonic $MgCl_2$ solution is slowly added to make 25% of the total volume. After 2 min. the decline of the frequency and amplitude of the rhythm is noticeable. At the same time the co-ordination of the rhythm between tentacles is progressively lost. Simultaneous beats of the tentacles become less frequent, and give way to contractions of greater amplitude and duration more characteristic of the spasm. Soon only temporary spasms of individual arms are seen. These spontaneous movements finally stop after 2 hr.

Meanwhile the co-ordination of the spasm between zooids is lost after a gradual decline, as tested at intervals by electrical stimulation on a part of the syndete not immersed in the solution. After 4 hr. the insensibility is complete, and when the head of an autozooid is cut off neither the head nor the stem contract. During recovery from magnesium the irregular movements of tentacles reappear first. These are replaced by an abnormally slow co-ordinated rhythm. The unco-ordinated movements do not merge into the rhythm, but for a time they are distinct as in normal life when a zooid is touched or shaken.

Calcium

The effect of excess calcium ions is primarily to slow the rhythm. No initial acceleration is found. In one particular colony counts of the rhythm gave initially twenty beats in $37{\cdot}3 \pm 2{\cdot}3$ sec. S.D. Addition of isotonic calcium chloride to make 1·2% and later 2·4% of the total volume was immediately followed by a slowing to twenty beats in 50 and 65 sec. respectively, from which there was some recovery after 15 min. in the mixture. On now increasing to 4·8% a spasm was produced in most autozooids, though occasionally there was some rhythm with long intervals of 5–10 sec. between beats. After 15 min. in the 4·8% solution there was some relaxation of the spasms and the zooids lay open with only occasional movements of single tentacles. The response of tentacles and zooids to touch and the co-ordination of the spasm over the colony remain normal long after the spontaneous rhythm has disappeared.

DISCUSSION

(*a*) *The co-ordination of the colony*

In some colonies a single shock produces no response, but a second at the same point within 3 sec. is followed by a wave of spasm. When the interval between the shocks is gradually reduced to less than 0·1 sec. the response suddenly disappears. With shocks well above threshold the minimum interval is of constant duration. It seems that we have here the absolute refractory period of the conducting system. In some coelenterates, of which *Calliactis* is the earliest example (Pantin, 1935), it is possible, by application of the second shock at a point distant from the first, to show that the whole pathway becomes refractory. This experiment is not successful with *Heteroxenia*, but at least the responding units include a link which works with an all-or-nothing action and the first excitation is a single discrete nerve impulse near its point of initiation. It is a suggestion that the excitation following the second stimulus is similar.

The durations of the spasms of the individual zooids are remarkably uniform over the colony and they recover their rhythm simultaneously. The excitation from the second shock has exactly the same effect as a single shock on the disk. It may be that a standard spasm is the only possible response, or this resemblance may imply that the conducting system brings a standard 'signal'. But observation soon eliminates a restriction of the response, for partial spasms and extra long spasms occur

frequently. A single shock to a disk always produces a symmetrical spasm whose duration is approximately constant for a range of intensities; but at high intensities or with several shocks, much longer spasms follow. All this suggests that the signal which reaches all the disks is a single impulse which in these colonies is initiated by the second shock on the syndete and is not a burst of impulses followed by a sensory accommodation. Variations in the spasm responses will then depend on local conditions and long spasms follow a sequence of impulses.

An observation on *Anthelia glauca* Savigny is relevant here. This is a xenid with no rhythm and the tentacles stand stiffly round the disk. A beautiful response is seen when condenser shocks at 30/min. stimulate the syndete. At each shock after the first the tentacles of all the forty or so zooids in the colony jerk simultaneously towards the mouth in a series of discrete contractions. The jerks correspond with the shocks and stop when they are discontinued.

(*b*) *The origin of the rhythm*

There is very little information about the conducting system which co-ordinates the beat. Artificial stimulation is here disappointing, for it produces only a spasm, never an extrasystole. The rhythm is reminiscent of the beat of medusae and the spasm is similar to the maintained contractions of the radial muscle of the ephyra larva of *Aurellia* (Horridge, 1955, Fig. 1) and of many Hydromedusae. Two lines of evidence suggest that the rhythm is similar. First, severed tentacles and parts of the disk show the rhythm, though slower than normal as though the part with

Table 1. *A summary of the reversible effects of ions on* Heteroxenia, Cassiopea *and* Rhizostoma

	Heteroxenia fuscescens	*Cassiopea andromeda*	*C. xamachana* from Mayer *Rhizostoma* from Bethe
Excess K^+ ions	Initial temporary spasms (indirect effect). Progressively slows then stops relaxed. Finally prevents transmission over colony	Initial acceleration (indirect effect). Progressively slows then stops. Finally prevents transmission in both nerve nets	Slows then paralyses. Oral arms paralysed (Mayer)
Excess Na^+ ions	Temporary spasms (indirect chemical stimulation). Accelerates the rhythm in strong solution. 10% produces paralysis in spasms	Temporary acceleration (indirect). Accelerates the rhythm	Weakly accelerates, then paralyses
Excess Ca^{2+} ions	Initial temporary spasms (indirect chemical stimulation). Progressively slows then stops the rhythm, followed by relaxed paralysis	Initial temporary acceleration (indirect). Progressively slows then stops	Momentarily accelerates then slows and then stops
Excess Mg^{2+} ions	Progressively slows then stops	Progressively slows then stops	Progressively slows then stops

(Paralysis≡not excitable and no transmission)

the fastest natural frequency drives the rest. The frequency of parts of various sizes support this (Fig. 4).

Secondly, *Heteroxenia* and the jellyfish *Cassiopea* (Rhizostomeae) react in a very similar way to excesses of potassium, sodium, calcium and magnesium ions if the primary effect on the rhythm alone is considered. But in *Cassiopea*, as in adult semaeostome medusae, excitation in the diffuse net accelerates the rhythm, whereas in *Heteroxenia* a spasm follows. Because of this difference the indirect effects on the rhythm contrast strongly. The results of earlier work (Mayer, 1906; Bethe, 1908) and my observations on *Heteroxenia* and on the Red Sea species *Cassiopea andromeda* are compared in Table 1.

The effect of an excess of the physiologically common cations on the rhythm of medusae, discovered by Loeb (1899), has been studied in detail by Mayer (1906, 1910) on *Cassiopea xamachana*, and Bethe (1908) on *Rhizostoma pulmo*. Mayer's theory of the origin of the rhythm has held the field for fifty years, but he argued that the rhythm arises from excess sodium in the ganglion because an access of sodium accelerates. Clearly this does not follow. The failure of excess external sodium to influence other nerve preparations leads to the conclusion that in coelenterates it acts by diluting the other ions.

The effects of ions are better known in medusae than in *Heteroxenia*, but the situation is confused and it is difficult to reach any conclusion. The results with some ions in excess of their normal concentrations can be interpreted in terms of the effects of these ions on the membrane potentials of other excitable tissue. Excess external potassium ions depolarize all excitable membranes known. For a rhythmical system this could increase the rate, or retard the repolarization of the membrane and decrease the rate. On various heart preparations excess potassium usually accelerates; but it usually slows down coelenterate rhythm. Excess calcium ions slow the rhythm of Purkinje fibres (Weidmann, 1955) by a rise of the threshold without change of resting potential, so that more depolarization is required to initiate an impulse. Excess calcium slows coelenterate rhythms and also a variety of heart preparations (data on hearts from Prosser, 1950, Table 69). According to Weidmann the effect of magnesium is similar to that of calcium. Excess sodium appears to have only small effects. In conclusion, the effects of ions on coelenterate rhythms suggest a polarized membrane mechanism, and in *Heteroxenia*, it is impossible to attribute the rhythm either to nerve or to muscle.

(*c*) *The two conducting systems of the disk*

The effects of ions on *Heteroxenia* suggest that the rhythm originates in a system with a polarized membrane as in nerve or muscle fibres. Two observations suggest the existence of separate systems of conduction for the beat and the spasm in the disk in addition to the contractile elements. First, in the beat the part with the fastest rhythm drives the whole. Therefore the excitation originates in the through-conducting pathway which conducts it. Secondly, there is the observation (Fig. 3D), that two opposite tentacles can show a co-ordinated rhythm while the intervening tentacles are in a spasm. The difficulty is to explain how the momentary

excitation of the beat can cross the regions of the disk where there is the excitation of the spasm. The inadequacy of a single conducting system in the disk rests on this observation, together with the generalization that in either a nerve net or a conducting membrane a long-lasting excitation travels further than a momentary one of the same kind. Also the spasm can be confined to one tentacle and also can be co-ordinated over the colony by a nerve impulse. The muscle fibres involved appear to be identical in both the local, maintained contraction of the spasm of a single tentacle, and the co-ordinated momentary contraction of the beat. It requires two lots of one-way transmission from two conducting systems to one set of muscle fibres (or alternatively to two sets of intermingled muscle fibres) to explain the observation that these two kinds of excitation remain distinct. The ephyra larva of *Aurellia* gives exactly comparable data, but the two conducting systems can be histologically demonstrated as two nerve nets (Horridge, 1955). In *Heteroxenia* two conducting systems are only inferred; the refractory period of part of one has been measured, the other reacts to common anions as a typical membrane conductor.

SUMMARY

1. In *Heteroxenia* there are two distinct kinds of response; one is the unco-ordinated rhythm of the autozooids; the other is the maintained spasm which is co-ordinated over the colony.

2. The absolute refractory period of the conducting system which co-ordinates the spasm of the colony is found to be 0·08 sec.

3. The actions of excesses of potassium, sodium, calcium and magnesium ions suggest that the rhythm resembles that of Scyphozoa.

4. The organization and overlap of the two conducting systems in the autozooid disk recall the scyphozoan pattern of two nerve nets. However, in *Heteroxenia* there is little evidence of interaction between the two systems.

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A STUDY OF THE OXYGEN CONSUMPTION OF FIVE SPECIES OF LEECH

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A correlation between the oxygen consumption of aquatic animals and their ecology has been demonstrated on many occasions (see Prosser, 1950, table 42; Whitney, 1942; Walshe, 1948; and Berg, 1952). Regarding fresh-water forms, two main conclusions emerge: (1) that many inhabitants of rapid streams have a higher rate of oxygen consumption than similar and closely related forms from slow streams or standing water; and (2) that inhabitants of oxygen-deficient water are often able to maintain a steady rate of oxygen consumption in the face of falling oxygen tension in the medium, until a critical level of oxygen tension is reached, below which the oxygen consumption falls rapidly.

This paper records a preliminary investigation into the oxygen consumption of five species of leech, *Glossiphonia complanata* (L.), *Helobdella stagnalis* (L.), *Erpobdella octoculata* (L.), *E. testacea* (Sav.) and *Piscicola geometra* (L.). An earlier study of the ecology of leeches (Mann, 1955) had shown that, while several of these species are widely distributed in various types of fresh-water habitat, each has a distinct habitat optimum, where it is the most abundant leech species and can be found in good numbers. *Glossiphonia complanata* thrives best in hard, fast-running water, but is by no means uncommon in lakes and ponds, and *Helobdella stagnalis* is abundant in hard, eutrophic lakes. *Erpobdella octoculata* occurs most frequently in soft-water streams, but it also survives better than other leeches in soft, peaty lakes and ponds. *E. testacea* is restricted in its distribution, and in the survey mentioned above its optimum habitat was not determined. Subsequent observations suggest that its preference is for overgrown situations, perhaps best described as reed swamps. *Piscicola geometra* may be found in the same situations as *Glossiphonia complanata*, viz. fast-running water, and it is rarely found in standing water, except in the surf zone of lakes.

These five species, selected primarily because they were easily available for experimental work, represent the three families Glossiphoniidae, Erpobdellidae and Ichthyobdellidae. Leeches of the first family typically have no haemoglobin in the blood, and no accessory respiratory organs. The Erpobdellidae have haemoglobin in the blood but no accessory respiratory organs, and the Ichthyobdellidae frequently (as in *Piscicola*) have pulsatile vesicles on the sides of the body. These vesicles are filled with colourless coelomic fluid and probably aid respiration.

METHODS

The concentration of dissolved oxygen was determined by polarography, a method in which a small voltage is applied between a dropping mercury electrode and a calomel electrode, and in which the resulting diffusion current is proportional to the concentration of oxygen in solution (for details see Kolthoff & Lingane, 1952). Guigère & Lauzier (1945) have shown that the concentration of oxygen in sea water can be determined polarographically to the nearest 0·02 ml./l., and Bartels (1949) showed that the curve relating diffusion current to oxygen concentration is a straight line passing through the origin if the applied voltage is carefully chosen. In certain solutions it is necessary to add substances such as gelatin, in order to suppress polarographic maxima, which have the effect of distorting the calibration curve. In the present work it was found that filtered water from Whiteknights Lake, Reading, could be used without the addition of a suppressing substance, and that the calibration curve relating concentration of dissolved oxygen to diffusion current was a straight line passing through the origin when the applied voltage was $-0{\cdot}55$ V. The original calibration curve was obtained by plotting the diffusion current against the concentration of dissolved oxygen as measured by the Winkler method. For subsequent calibration of the instrument it was only necessary to determine the diffusion current for air-equilibrated water (containing 6·35 ml. oxygen per litre at 20° C. and 760 mm. pressure), and then join this point to the origin.

This electrical method has the advantage over chemical methods of greater rapidity, of comparable or greater accuracy, and of permitting a continuous record of changes in oxygen concentration to be obtained if required.

It was established that the leeches could live for many days in contact with mercury (even if, as sometimes occurred, they swallowed beads of mercury) and that the presence of mercury has no discernible effect on their oxygen consumption.

To determine the oxygen consumption for a particular species at a particular oxygen concentration, the leeches were sorted according to size into about five small glass-stoppered bottles, the number of leeches and the size of bottle being chosen so that the leeches produced a drop of 10–20% in the concentration of oxygen when kept at $20 \pm 0{\cdot}1°$ C. for about 1 hr. The activity of the leeches was kept at a minimum by wrapping the respiration bottles in black paper. The oxygen concentration was determined polarographically both before and after the period of oxygen uptake, and the leeches were then removed and weighed alive after drying surface moisture on filter-paper. In all experiments, other than those in §C below, care was taken to acclimatize the leeches to a temperature of 20° C. for at least 24 hr., and usually for 36–48 hr. During temperature acclimatization they were kept in well-aerated water.

EXPERIMENTS

A. *Oxygen consumption in relation to size*

The difficulty of obtaining the leeches in large numbers made it desirable to use those of every size group, so the relationship of oxygen consumption to size had

first to be investigated. Fig. 1 shows the results of forty determinations of oxygen consumption of *Glossiphonia complanata* in air-saturated water at 20° C. Each point represents the mean oxygen consumption of two to six leeches enclosed in a bottle of capacity 6–7 ml. for 1–1½ hr. The equation of the regression line is

$$\log r = 0{\cdot}715 \log W + \log k,$$

where r = the mean rate of oxygen consumption, W = the mean weight of the leeches and k is a constant. This may also be written $r = kW^{0{\cdot}715}$ indicating that the rate of uptake is roughly proportional to the surface area rather than to the mass.

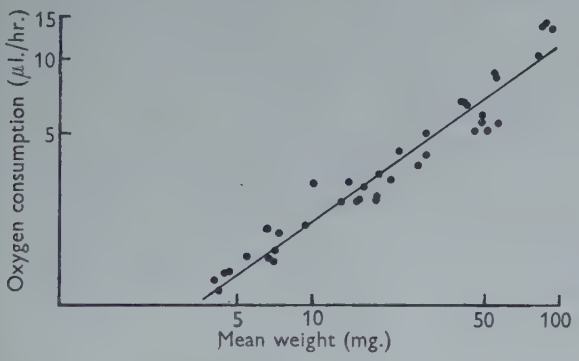

Fig. 1

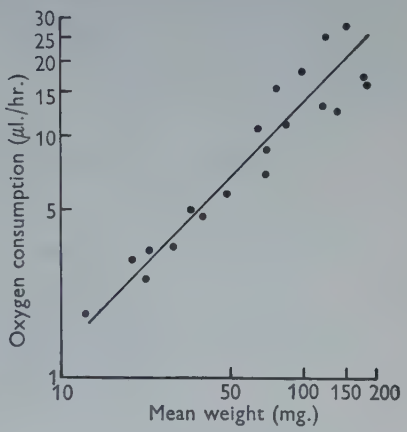

Fig. 2

Fig. 1. Oxygen consumption of *Glossiphonia complanata* in air-saturated water at 20° C., plotted as a function of live weight.

Fig. 2. Oxygen consumption of *Erpobdella octoculata* in air-saturated water at 20° C., plotted as a function of weight.

Table 1. *Slope (m) of the line relating oxygen concentration to weight for five species of leech*

Species	Weight range (mg.)	*m*
Glossiphonia complanata	4–94	0·715
Helobdella stagnalis	2–6	0·81
Piscicola geometra	2–22	0·695
Erpobdella testacea	6–37	0·81
E. octoculata	12–104	1·06

Table 1 shows the results of similar determinations of the slope of the line relating log r to log W, for the other species investigated. In every case the slope of the line is greater than two-thirds, the most remarkable being *Erpobdella octoculata* where it is greater than unity (Fig. 2.).

B. *Comparison of the oxygen consumption of the five species*

The rates of oxygen uptake were compared in the five species of leech, under the conditions described above. Observations made through gaps in the black paper

covering suggested that the leeches were reasonably inactive while the oxygen concentration was at or near that of air-saturated water, so that the figures obtained represent something approaching the basal or standard metabolic rate. Owing to the wide range of sizes of leeches used, it was not possible to arrive at a representative figure for oxygen uptake in terms of ml./g./hr. For example, Fig. 1 shows that with *Glossiphonia complanata* the rate of uptake varies from 286 μl./g./hr. for a small leech, to 102 μl./g./hr. for a large one. Comparison was therefore made on the basis of the oxygen consumption of a leech of 30 mg. This figure can be obtained for each species from the line relating oxygen consumption to weight, either directly or by extrapolation. The values for the various species are given in Table 2. The outstanding feature is that *Piscicola geometra* has a much higher oxygen consumption than the other leeches.

Table 2. *Oxygen consumption of a leech of* 30 *mg. at* 20° C. *in air-saturated water*

Species	Oxygen consumption (μl./hr.)
Glossiphonia complanata	4·95
Helobdella stagnalis	6·10
Piscicola geometra	10·10
Erpobdella testacea	5·98
E. octoculata	4·0

C. *The effects of starvation*

Specimens of *Glossiphonia complanata* were collected from the River Pang near Bradfield, Berkshire, and estimations of the oxygen consumption were made at intervals over a period of 7 days. (Each estimation was based on five measurements, as explained previously.) The method used to calculate the oxygen consumption of a leech of 30 mg. was a graphical one suggested by Prof. Kaj Berg. The mean oxygen consumption for the leeches in each bottle was plotted against their mean weight on a logarithmic scale, and the line of best fit to the five points thus obtained was drawn in, keeping the slope of the line at the value previously determined for the species, and varying only the point of intersection of the line with the Y axis. For example, it was shown in §A above that the slope of the line relating oxygen consumption to weight in *G. complanata* is 0·715. The five points shown on Fig. 3 were obtained by the methods described above, and the line AB was drawn through the origin to make an angle $\tan^{-1}$ 0·715 with the X axis. The line CD was drawn parallel to AB at the level which provided the best fit for the five points. The oxygen consumption for a leech of 30 mg. is seen to be 5·2 μl./hr.

The results of measuring the oxygen consumption under conditions of starvation are shown in Table 3. The standard error of the intercepts on the graphs relating respiration to weight at different times was calculated, and the differences between them subjected to the 't' test. It was found that the variations in oxygen consumption were without significance. It was therefore concluded that it is possible to

experiment with *G. complanata* for at least 7 days without the results being affected by starvation. The same conclusion was reached in respect of the other species.

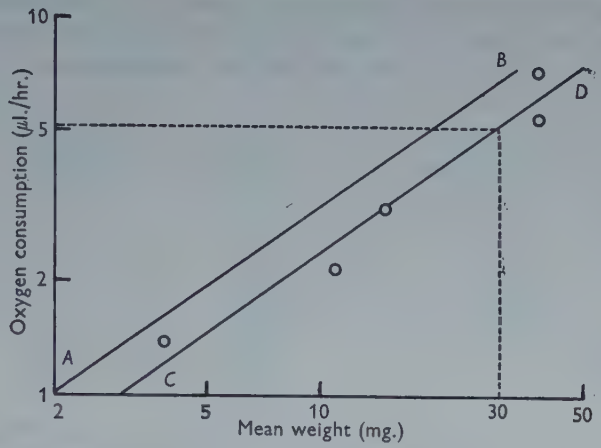

Fig. 3. Diagram illustrating graphical method of calculating the oxygen uptake of a leech of standard weight. For explanation see text.

Table 3. *Oxygen consumption of starved* Glossiphonia complanata *of* 30 *mg. at* 20°C. *in air-saturated water*

	Time in laboratory after collection						
	1·5 hr.	4·75 hr.	6·5 hr.	10·5 hr.	23·5 hr.	3 days 5 hr.	7 days
Oxygen consumption (μl./hr.)	5·2	5·6	4·8	4·6	4·2	4·4	5·2

D. *Oxygen consumption in relation to oxygen tension*

The oxygen consumption of each species was determined by the methods described above, but the concentration of dissolved oxygen was reduced before the start of each experiment. The routine was as follows. Nitrogen was bubbled through Whiteknights water until the oxygen tension, as determined polarographically, had fallen to the required level. Five bottles containing leeches, and two or three empty bottles were then filled with this water, closed, and placed in the water-bath for 1 hr. At the end of this period, the oxygen concentration in the bottles without leeches was taken as the value at the beginning of the experiment, and the concentration in the bottles containing leeches was used to calculate their oxygen consumption. The value for a leech of 30 mg. was plotted against the mean oxygen concentration during the experiment, and the curves thus obtained are illustrated in Fig. 4. The three species *Erpobdella octoculata*, *E. testacea* and *Piscicola geometra* show clearly the dependence of oxygen consumption on oxygen tension. *Helobdella stagnalis* shows a degree of independence between 2.0 and 4·0 ml./l., and *Glossiphonia complanata* appears to be independent between 3·5 and 6·0 ml./l., but it may be noted that the maximum value recorded is well below

that shown in Fig. 1 for conditions of air-saturation. The experiments on *G. complanata* with reduced oxygen concentrations were made in December, while the earlier experiments were carried out in September, and it may be that the form of the curve in summer is that indicated by the dotted line, and the effect of winter conditions is to depress the line at higher oxygen concentrations.

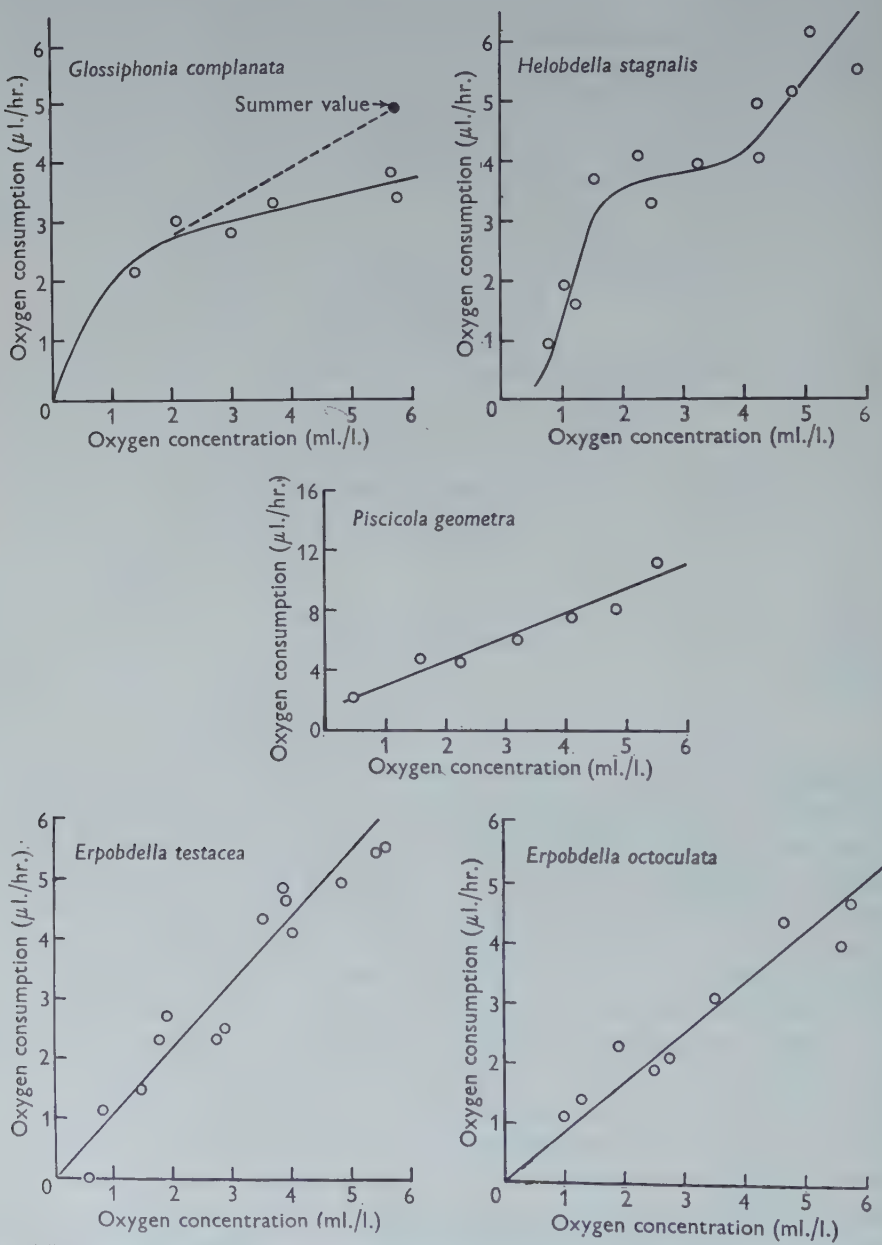

Fig. 4. The relation between oxygen consumption and oxygen concentration for five species of leech, at 20° C., with no acclimatization to low oxygen tensions.

E. *The effect of acclimatization to low oxygen concentration*

The measurements made in the previous section were repeated, but instead of the leeches being taken from air-saturated water and placed directly in the respiration bottles, they were placed overnight in water of the oxygen concentration required for the experiment. For instance, in one experiment ten leeches were placed in the evening in 2 l. of water having an oxygen concentration of 4 ml./l., and this was covered with liquid paraffin. By morning the concentration had fallen to 3·75 ml./l. and this water was then used for determining oxygen consumption by

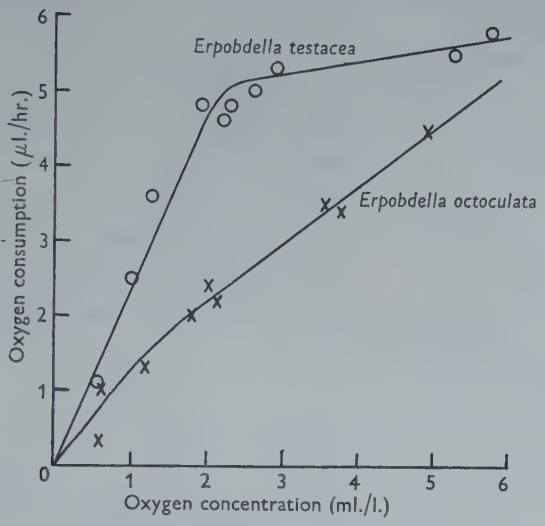

Fig. 5. The relation between oxygen consumption and oxygen concentration in the two species of *Erpobdella*, after acclimatization overnight to the concentration of oxygen at which the readings were taken.

the method described above. All the species except *Erpobdella testacea* showed the same relationship between oxygen consumption and oxygen concentration as before, but *E. testacea* now showed a high degree of independence, as illustrated in Fig. 5., there being little change in the oxygen consumption between 6·0 and 3·0 ml./l. oxygen.

F. *Oxygen consumption and behaviour of* Erpobdella Testacea *when confined in a small volume of water*

Four leeches were placed in a respiration bottle, 15 ml. of air-saturated water at 20° C. were added, and the electrodes of the polarograph were dipped into it, after which liquid paraffin was poured over the surface of the water. The fall in oxygen concentration is recorded in Fig. 6. Observations on the behaviour are noted beside the line. It is seen that when the oxygen concentration fell to about 4 ml./l. the leeches began to undulate their bodies. This activity caused a current of water to flow past the body surface and the increased activity, together with the

increased ventilation, resulted in a rapid fall in the oxygen concentration, i.e. an increased uptake by the leeches. At about 1·5 ml./l. the pumping became less frequent, and was soon stopped. There was then a corresponding fall in their rate of uptake.

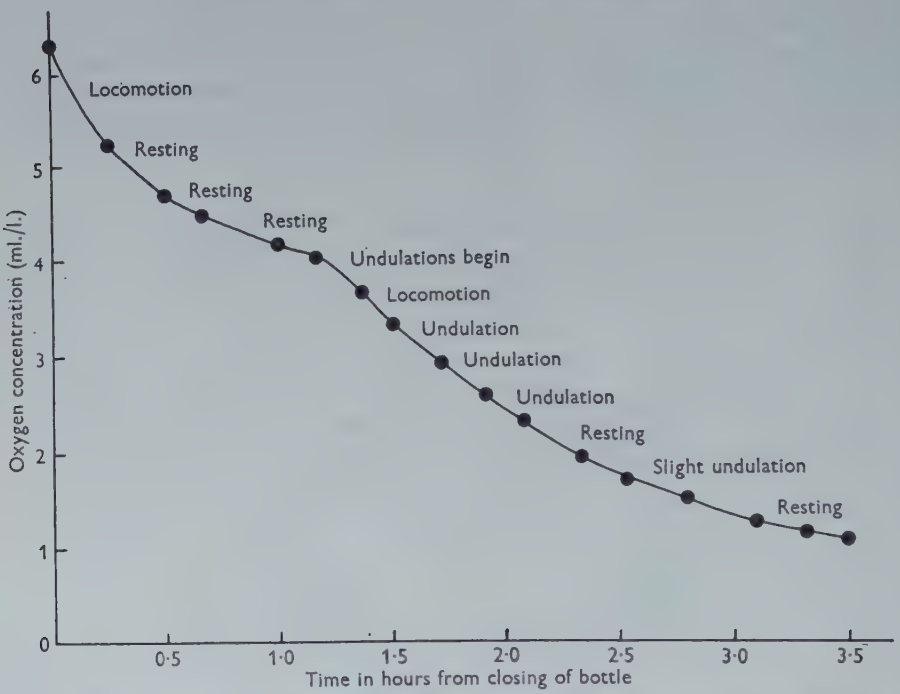

Fig. 6. Change in oxygen concentration with time, when four specimens of *Erpobdella testacea* were enclosed in 15 ml. of air-saturated water at 20° C. Notes refer to type of behaviour.

G. *Rate of respiration of* Erpobdella testacea *at low temperature* (0·4° C.) *with and without acclimatization*

The previous experiment suggested that the respiratory independence of *E. testacea* illustrated in Fig. 5 might be produced by muscular ventilating activity rather than by an internal physiological mechanism, so experiments D and E were repeated at 0·4° C., at which temperature there were almost no muscular movements. The results, illustrated in Fig. 7, show that there was little difference between the oxygen uptake with and without acclimatization, suggesting that the acclimatization effect previously observed was brought about by the leech ventilating its body surface by undulation.

DISCUSSION

It is interesting to compare the results of the study of oxygen consumption in relation to weight with those of Whitney (1942) for fresh-water Turbellaria. He, too, found that certain species fitted the surface law of Rubner (1883) (as extended to poikolothermous forms by Voit, 1901), i.e. the oxygen consumption, r, was

related to the weight, W, according to the formula $r = kW^{\frac{2}{3}}$. On the other hand, he found that some species departed widely from the law. For *Polycelis nigra* he found that the oxygen consumption per unit weight was actually lower in the smaller animals, and in the present study the same is true for *Erpobdella octoculata*, where $r = kW^{1 \cdot 06}$. It appears from Prosser (1950) that the cause of the normal relationship between body weight and oxygen uptake is obscure, so there seems little point in speculating as to the reasons for departures from the rule.

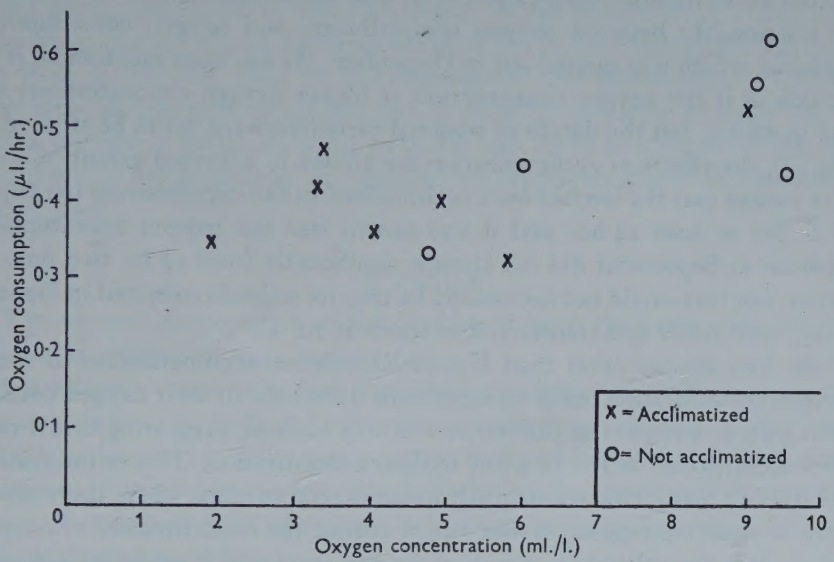

Fig. 7. The relation between oxygen concentration and oxygen consumption in *Erpobdella testacea* at 0·4° C.

The outstandingly high rate of oxygen consumption of *Piscicola geometra* is in accordance with expectations for three reasons. First, it is the only leech studied which does not rely entirely on diffusion of oxygen through the general body surface. Its pulsatile vesicles are thin-walled bulges from the sides of the body. They are in direct communication with the coelomic lacunar system, and are equipped with valves so that their pulsations bring about a one-way circulation of coelomic fluid (Selensky, 1915). *Piscicola* then, has the advantage over the other leeches of an organ system which brings coelomic fluid into proximity with the water, and then pumps it round the body. Secondly, *Piscicola* feeds by attaching itself to passing fish, and sucking their blood. During the period when it is actively seeking a host it has to perform extremely rapid movements, and one would expect there to be an efficient system of gaseous exchange if only for use at this time. Thirdly, *Piscicola* is characteristically a stream form, and as has been previously mentioned, there is an increasing body of evidence for the view that stream forms normally have a higher oxygen consumption than pond or lake animals.

The differences in level of oxygen consumption among the remaining species are not very great, and are probably not significant if one takes into account the

possibility that the level of activity of the different species may vary slightly under the conditions of the experiment.

The relation of oxygen consumption to oxygen concentration for the various species is still far from clear owing to the large number of variables in the situation. Perhaps the two most important in the present study are (i) seasonal variation in oxygen consumption, and (ii) the effects of acclimatization to the temperature and oxygen concentration of the experiment. Regarding (i), seasonal variation, all the work was carried out in the months April-September, with the exception of the determination of the relationship between oxygen concentration and oxygen consumption in *Glossiphonia*, which was carried out in December. As has been mentioned, it looks very much as if the oxygen consumption at higher oxygen concentrations is depressed in winter, but the details of seasonal variations have yet to be studied. Regarding (ii), the effects of acclimatization are known to a limited extent. Care was taken to ensure that the leeches were acclimatized to the experimental temperature of 20° C. for at least 24 hr., and it was shown that the oxygen consumption of *Glossiphonia* in September did not change significantly from $1\frac{1}{2}$ hr. to 7 days after collection; but this would not necessarily be true for animals collected in December from very cold water and transferred to water at 20° C.

For the four species other than *Erpobdella testacea*, acclimatization to water of low oxygen concentration made no significant difference to their oxygen consumption, but with *E. testacea* this difference was very marked, suggesting that it rapidly becomes acclimatized to life in a low oxygen concentration. This is interesting in view of the fact that it characteristically inhabits reed swamps where there are large amounts of decaying organic matter which reduce the concentration of oxygen in the water. It is also relevant to note that the two species of *Erpobdella* have haemoglobin in the blood, and from work on other annelids (Johnson, 1942; Fox, 1945) one would expect this to assist oxygen uptake, at least at low oxygen tensions. There is, however, no evidence that the haemoglobin affected oxygen uptake in the present experiments. Preliminary observations on the ventilation activity of *Erpobdella testacea* suggest that this plays an important part in maintaining a steady rate of oxygen consumption in the face of falling oxygen tension, and the fact that there was no difference in the oxygen consumption of leeches acclimatized and leeches not acclimatized when ventilation was inhibited by low temperatures supports this idea. If it is correct, then the difference between the oxygen consumption of the two species of *Erpobdella* at low oxygen tensions after acclimatization must be related to their different patterns of behaviour under these conditions. This aspect requires further study.

It has also been noticed that *Helobdella* makes ventilatory movements under conditions of reduced oxygen concentration. In Whiteknights Lake, from which these animals were collected, it is not unusual for the concentration of oxygen at a little distance from the surface to fall to below 4·0 ml./l. in summer. It is suggested that when this happens the leeches respond by making ventilatory movements which keep the oxygen consumption at a steady level, unless the concentration falls below 2 ml./l. It is customary to assign to various animals a critical tension of O_2, t_c,

below which oxygen consumption falls rapidly, but above which oxygen consumption is relatively independent of the oxygen tension. Hyman (1929) has shown how acclimatization can alter the critical concentration for *Planaria* from 3 to 0·5 ml./l., and there is little doubt that statements regarding t_c values ought always to be qualified by details of previous acclimatization. In many experiments where animals have been confined in a vessel and allowed to reduce the oxygen concentration slowly (e.g. Hiestand, 1937), it is probable that some acclimatization occurred during the course of the experiment. In the present work a clear distinction has been made between experiments in which no acclimatization was possible (other than during the hour of the experiment) and experiments in which the animals were acclimatized overnight, but it would be desirable to know the effect of longer periods of acclimatization, or better still, to have a record of the oxygen consumption under conditions of constant low oxygen tension over a considerable period of time.

SUMMARY

1. The oxygen consumption of five species of leech has been investigated and considered in relation to their ecology.

2. *Glossiphonia complanata* and *Erpobdella octoculata* which are most common in, but not confined to, hard and soft water streams respectively, have their oxygen consumption dependent on the concentration of dissolved oxygen, at least in spring and summer. Their oxygen uptake is not affected by acclimatization overnight to a low level of oxygen, but the uptake of *Glossiphonia* at the higher oxygen concentrations is depressed in winter.

3. *Erpobdella testacea* has an oxygen consumption which is independent of the oxygen concentration between 6·0 and 3·0 ml./l., provided that the leeches have been acclimatized overnight to the oxygen concentration at which their uptake is measured. Ventilation of the body surface by dorso-ventral undulations appears to be an important factor in the maintenance of a high rate of oxygen uptake at low concentrations. This species is found in reed swamps.

4. *Helobdella stagnalis*, which is most abundant in stagnant eutrophic lakes, maintains a level of oxygen consumption which is independent of the oxygen concentration between 2·0 and 4·0 ml./l., even without previous acclimatization.

5. *Piscicola geometra*, which is virtually absent from stagnant water, has a higher rate of oxygen uptake than any of the other species under conditions of air-saturation, and its rate is strictly dependent on the concentration of oxygen in the water.

The techniques employed in this work were mostly suggested by Prof. Kaj Berg, of the University of Copenhagen Freshwater Biological Station, Hillerød, Denmark, to whom the author is greatly indebted for his interest and hospitality. Thanks are also due to Prof. A. Graham for his encouragement and helpful suggestions, and for criticizing the manuscript. The polarographic apparatus was purchased from a grant by the Research Board of the University of Reading.

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